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USAF RADIATION LABORATORY

QUARTERLY PROGRESS REPORT

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USAF

RADIATION LABORATORY

QUARTERLY PROGRESS REPORT NO. 35

APRIL 15, 1960

Contract No. AF 41(657)-252

A contract between the University of Chicago and the School of Aviation Medicine, Air University Command, United States Air Force for research on certain biological and medical aspects of atomic energy.

Kenneth P. DuBois, Director

TABLE OF CONTENTS

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TABLE OF CONTENTS--Continued

THE EFFECTS OF IONIZING RADIATIONS ON THE BIOCHEMISTRY OF MAMMALIAN TISSUES

I. The Effects of Radioprotective Agents on the Glutathione Content of Animal Tissues

Gerald B. Zina, Ann D. Raymond and David M. Seidel

This report concerns: The influence of radioprotective agents on the glutathione levels of the tissues of rats and mice. This study constitutes one phase of a detailed investigation of the mechanism of the toxic and radioprotective actions of chemical agents.

Immediate or ultimate application of the results: The development of effective therapeutic or prophylactic agents for radiation injury would be facilitated by an understanding of the mode of action of those compounds which are known to exhibit radioprotective activity. With this objective in mind detailed studies on the mode of action of aminoethylisothiourea derivatives and dithiocarbamates are underway in this laboratory at the present time. As a part of this program measurements of the influence of 2-aminoethylisothiourea on the levels of reduced glutathione in animal tissues were recently undertaken and evidence was obtained that this compound causes considerable decreases in the reduced glutathione levels of some tissues. The present experiments were undertaken to ascertain whether various other radioprotective agents produce similar effects. The results of the measurements have indicated that several of the compounds decrease the glutathione levels of some tissues of rats and mice. At the present time it cannot be stated whether this action is involved in the radioprotective activity of the compounds or their toxicity. However, the results demonstrate that treatment of animals with various radioprotective agents causes some decrease in reduced glutathione levels in most cases. It is probable that this effect is due to a direct interaction between glutathione and the protective agents in some cases and that indirect mechanisms are responsible for the changes in the other cases.

* * * * * * * * *

Previous studies in this laboratory have demonstrated that 2-aminoethylisothiourea (AET) inhibits the activity of several sulfhydryl enzymes (1). In view of the ease with which AET forms mixed disulfides with simple thiols and disulfides in vitro (2) and with proteins in vitro and in vivo (3-5), it seems likely that the inhibition is the result of mixed disulfide formation with essential sulfhydryl groups of enzymes. The possibility that mixed disulfide formation is involved as a mechanism of radioprotection through temporary shielding of essential sulfhydryl groups of cellular constituents has been considered (2) but direct evidence in support of this idea is lacking. However, it seems likely that the toxic effects of AET and related compounds are due to reaction with essential sulfhydryl groups in the species which have been studied to date (1).

In an effort to obtain further information on the effects of AET on sulfhydryl compounds additional studies were recently conducted in this laboratory (6,7) in which the effect of AET on the glutathione levels of tissues of rats and mice was measured. The results indicated a close correlation between the ability of AET to decrease the glutathione levels of several tissues in the rat and mouse and the relative toxicity of the drug to these species. A number of investigators have measured the free sulfhydryl levels of tissues of irradiated animals (8-11). However, most of the measurements were performed on blood and liver rather than radiosensitive tissues and the methods employed measured total sulfhydryl groups. In contrast this study and our previous experiments utilized the specific glyoxylase method (12) which measures reduced glutathione exclusive of other sulfhydryl compounds.

Our previous studies (6,7) demonstrated that AET produces a dose-dependent decrease in the reduced glutathione levels of the liver, kidney and erythrocytes of rats and smaller changes in the spleen and brain. A similar effect was noted in mouse tissues with the exception that a more pronounced decrease was observed in brain. Exposure of rats to 900 r of whole body x-irradiation caused decreases in the reduced glutathione content of the red cells, thymus and intestine (6). In view of the ability of AET to decrease the levels of reduced glutathione in animal tissues, it was of interest to measure the effects of other compounds capable of protecting against radiation lethality on the glutathione levels of the tissues of rats and mice. The present report contains the results of measurements of the glutathione levels of several tissues after treatment of animals with various agents which have been shown to afford protection against radiation lethality. The results of these measurements demonstrated that several of the compounds cause significant decreases in the tissue levels of reduced glutathione.

Materials and Methods. Young adult, female Sprague Dawley rats and Carworth Farms mice were used for these experiments. The animals were housed in air-conditioned rooms and were maintained on Rockland Diet. Total body x-irradiation was administered with a G. E. Maximar Therapy Unit. The radiation factors were as follows: 250 KVP, 15 ma., 0.25 mm. Cu and 1 mm. Al filtration. The target distance was 75 cm. and the dose rate was 36 r to 41 r per minute measured in air.

Reduced glutathione was measured by the method of Woodward (12) using a yeast suspension prepared by the procedure of Albert et al. (13). Electrolytic reduction of the tissue filtrates to convert the oxidized glutathione to the reduced form was carried out by the procedure of Doman and Woodward (14). A detailed description of the preparation of various reagents used for the assay have been presented in previous reports from this laboratory (6,7).

Experimental

Effects of various radioprotective agents on the reduced glutathione levels of rat tissues. The effects of various radioprotective agents on tissue levels of reduced glutathione were measured by the conduction of assays on erythrocytes, spleen, liver, thymus and ileum of female rats. The drugs were given by the intraperitoneal route at doses near the maximum tolerated levels as determined by previous toxicity tests. Groups each containing at least three animals were sacrificed at thirty minutes and three hours after

[illegible] of the drugs. The results of these measurements are summarized in [illegible] in which the average values and the average deviations from the mean are presented together with the per cent change in reduced glutathione levels.

As shown by the data in Table 1 several of the compounds produced moderate changes in the reduced glutathione levels of one or more tissues of rats. Thus 2-diethylaminopropiophenone caused a 39% decrease in the glutathione concentration of erythrocytes in thirty minutes and changes of a similar magnitude in liver and ileum at three hours. Cyanide caused a 27% decrease and hydroxyacetonitrile caused a 62% decrease in the reduced glutathione content of the liver in three hours. Histamine, cysteine and hydroxylamine caused some reduction of the glutathione levels of all of the tissues in the red blood cells and liver. Reduction of the glutathione levels of all of the tissues was observed after treatment with 5-hydroxytryptamine with the effect of this drug being most pronounced in the liver at three hours after administration. AET, mercaptoethylamine and dimethylammonium dimethyldithiocarbamate also produced marked decreases in the glutathione content of the liver and some decreases in other tissues. Exposure of rats to a simulated altitude of 30,000 feet caused a 43% increase in the reduced glutathione content of the erythrocytes and a [illegible] 47% decrease in the liver.

Effect of various radioprotective agents on the reduced glutathione levels of mouse tissues. The influence of various radioprotective agents on the reduced glutathione content of the kidney, spleen, liver and ileum of mice was measured at one and three hours after intraperitoneal injection of maximum tolerated doses of the compounds. The results of these measurements are summarized in Table 2 in which the average reduced glutathione content for groups of six animals are presented together with the average deviation from the mean and the per cent change in levels as compared with normal animals.

The data in Table 2 indicate that most of the compounds employed for this study have no appreciable effect on the reduced glutathione content of mouse tissues. Less than 25% reduction of the normal levels were observed after treatment with AET, hydroxyacetonitrile, cysteine and hydroxylamine. 2-diethylaminopropiophenone caused 27% reduction in the spleen and 20% reduction in the liver in three hours and histamine caused changes of a similar magnitude in [illegible] and liver. The dimethylammonium salt of dimethyldithiocarbamate caused [illegible] reduction of the glutathione content of the kidney and [illegible] reduction in the liver and 5-hydroxytryptamine caused a 42% decrease in the glutathione content of the kidney. The results of these measurements indicated that most radioprotective agents have a pronounced influence on the reduced glutathione levels of mouse tissues. Previous studies in this laboratory have shown (1) that AET at the same dosage level has more pronounced effects on certain sulfhydryl enzymes than on reduced glutathione and similar results are now being obtained with dimethylammonium dimethyldithiocarbamate.

Influence of various radioprotective agents on the levels of oxidized and reduced glutathione in the tissues of male rats. In the experiments described above the reduced glutathione content of the tissues was measured because it was anticipated that effects of the various drugs would be more likely to involve oxidation or interaction with reduced glutathione than with the oxidized form. To obtain information in connection with this assumption measurements of the oxidized and total glutathione were performed on liver, spleen and

TABLE 1

EFFECT OF VARIOUS RADIOPROTECTIVE AGENTS ON THE REDUCED GLUTATHIONE LEVELS OF RAT TISSUES

Dose of Drug (mgm /kgm.)	Hours After Drug	Mg. % Reduced Glutathione				
		RBC	Spleen	Thymus	Liver	Ileum
Control						
....	....	56 ± 5	115 ± 3	89 ± 7	225 ± 12	100 ± 4
p-Aminopropiophenone						
30	0.5	34 ± 5 -39%	106 ± 5 -8%	96 ± 7 +8%	200 ± 20 -11%	100 ± 12 0%
	3.0	58 ± 6 +4%	100 ± 4 -13%	92 ± 14 +3%	138 ± 7 -39%	84 ± 3 -21%
Potassium Cyanide						
3	0.5	58 ± 3 +4%	117 ± 7 +2%	98 ± 9 +1%	222 ± 8 -1%	102 ± 13 +4%
	3.0	57 ± 8 +2%	110 ± 6 -4%	92 ± 14 +3%	166 ± 11 -27%	93 ± 15 -12%
Hydroxyacetonitrile						
7.5	0.5	66 ± 6 +18%	115 ± 2 0%	112 ± 6 +26%	225 ± 10 0%	108 ± 8 +2%
	3.0	64 ± 5 +14%	105 ± 8 -9%	81 ± 3 -9%	86 ± 14 -62%	80 ± 3 -20%
Histamine Dihydrochloride						
425	0.5	47 ± 6 -16%	119 ± 4 +4%	89 ± 1 0%	205 ± 23 -9%	102 ± 10 +4%
	3.0	41 ± 2 -27%	102 ± 2 -11%	86 ± 5 -3%	158 ± 4 -30%	83 ± 3 -22%
Cysteine Hydrochloride						
1000	0.5	34 ± 2 -39%	118 ± 5 +2%	100 ± 9 +2%	228 ± 9 0%	100 ± 1 0%
	3.0	54 ± 7 -4%	111 ± 4 -3%	100 ± 9 +12%	235 ± 6 +4%	95 ± 1 -10%

TABLE I.--Continued

Dose of Drug (mgm./kgm.)	Hours After Drug	Mg. % Reduced Glutathione				
		RBC	Spleen	Thymus	Liver	Ileum
5-Hydroxytryptamine Creatinine Sulfate						
150	0.5	57 ± 7 +2%	93 ± 6 -19%	71 ± 7 -20%	193 ± 3 -14%	95 ± 1 -10%
	3.0	45 ± 7 -20%	67 ± 1 -42%	75 ± 10 -16%	72 ± 2 -48%	72 ± 10 -32%
Hydroxylamine Hydrochloride						
75	0.5	53 ± 4 -5%	112 ± 1 -3%	104 ± 2 +17%	207 ± 9 -8%	110 ± 2 -4%
	3.0	47 ± 2 -16%	101 ± 5 -12%	92 ± 3 +3%	157 ± 6 -30%	96 ± 4 -9%
2-Aminoethylisothiuronium Dichloride						
175	0.5	63 ± 6 +13%	109 ± 7 -5%	90 ± 3 +1%	154 ± 9 -32%	100 ± 4 -6%
	3.0	59 ± 8 +5%	105 ± 2 -9%	95 ± 5 +6%	121 ± 10 -46%	74 ± 9 -30%
Mercaptoethylamine Hydrochloride						
200	0.5	67 ± 9 +20%	116 ± 11 +1%	.....	211 ± 2 -6%	.....
	3.0	57 ± 1 +2%	100 ± 9 +4%	87 ± 6 -2%	96 ± 24 -57%	91 ± 7 -14%
Dimethylammonium Dimethyldithiocarbamate						
400	0.5	18 ± 6 -68%	107 ± 2 -7%	.....	185 ± 15 -18%	.....
	3.0	47 ± 13 -16%	99 ± 4 -14%	96 ± 7 +7%	135 ± 9 -40%	110 ± 4 +3%
Reduced Glutathione						
900	3.0	58 ± 0 +4%	101 ± 1 -12%	93 ± 1 +4%	159 ± 1 -14%	102 ± -4%
Altitude (30,000 feet)						
3.0 hrs.	0-0.5	80 ± 2 +43%	120 ± 10 +4%	104 ± 4 +17%	120 ± 4 -42%	82 ± 6 -23%

Table 2

[illegible]

Dose of Drug (mgm./kgm.)	Hours After Drug	Mg. % Reduced Glutathione			
		Kidney	Spleen	Liver	Blood
Control					
....	...	117 ± 11	123 ± 6	281 ± 12	112 ± 7
2-Aminoethylisothiuronium Dichloride					
300	3	101 ± 4 -15%	110 ± 6 -11%	232 ± 12 -17%	94 ± 2 -16%
[illegible]antoethylamine Hydrochloride					
200	1	114 ± 5 -3%	134 ± 5 +9%	219 ± [illegible] -22%	109 ± 10 -3%
	3	101 ± 9 -14%	116 ± 8 -6%	184 ± 13 -35%	90 ± 2 -20%
-[illegible]-piophenone					
39	1	101 ± 1 -14%	131 ± 4 +7%	244 ± [illegible] -13%	100 ± 6 -11%
	3	85 ± 1 -27%	130 ± 7 +6%	268 ± 12 -5%	90 ± 5 -20%
Hydroxyacetonitrile					
7.5	1	.....	107 ± 5 -13%	279 ± 15 -1%	107 ± 14 -4%
	3	94 ± 1 -20%	119 ± 2 -3%	261 ± 2 -7%	104 ± 3 -7%
Cysteine Hydrochloride					
1000	1	105 ± 3 -10%	116 ± 3 -6%	288 ± 19 +2%	93 ± 5 -17%
	3	92 ± 1 -21%	117 ± 5 -5%	275 ± 18 -2%	105 ± 4 -2%

TABLE 2--Continued

Dose of Drug (mgm./kgm.)	Hours After Drug	Mg. % Reduced Glutathione			
		Kidney	Spleen	Liver	Ileum
Histamine Dihydrochloride					
425	3	87 ± 6 -26%	117 ± 2 -5%	226 ± 24 -20%	93 ± 4 -17%
5-Hydroxytryptamine Creatinine Sulfate					
150	3	69 ± 4 -42%	122 ± 3 -1%	253 ± 3 -10%	102 ± 1 -9%
Hydroxylamine Hydrochloride					
75	3	99 ± 8 -15%	116 ± 3 -6%	279 ± 25 -1%	104 ± 3 -7%
Dimethyl[illegible]onium Dimethyldithiocarbamate					
600		89 ± 4 -24%	115 ± 2 -7%	267 ± 11 -5%	7[illegible] - 6 -36%

ileum of rats after treatment with some of the protective agents. In these measurements total glutathione consisted of the amount of reduced glutathione existing in the tissue filtrates after electrolytic reduction which was carried out according to the procedure of Boban and Woodward (14). The results of these measurements are summarized in Table 3 in which the average values for groups containing at least three animals are presented together with the average deviation from the mean. The results of these determinations demonstrated that there was no significant increase in reduced glutathione content of the tissue filtrates after electrolytic reduction and that the protective agents had the same effect on the reduced glutathione levels before and after electrolytic reduction. Assays conducted on red blood cells, thymus and ileum of rats after exposure to 800 r of x-ray showed that the same amount of inhibition occurred with or without electrolytic reduction of the glutathione.

Discussion

The results of the present investigation have shown that several radioprotective agents cause decreases in the reduced glutathione levels of the tissues of rats and mice. However, in most cases the amount of reduction amounted to less than 25%. Substantial decreases in the reduced glutathione levels of liver were observed after treatment with hydroxyacetonitrile, cysteine and 5-hydroxytryptamine. All of these compounds had less effect on the glutathione content of the tissues of mice than was observed in rats. Previous studies in this laboratory (1) on the influence of AET on enzyme systems have indicated that sulfhydryl enzymes are affected to an equal or greater extent than the reduced glutathione levels. This finding is of considerable interest since some attention has been given to the possibility of measuring the reactivity of sulfur-containing radioprotective agents in terms of their ability to react with glutathione. The results of the present experiments, as well as other experiments in progress on the actions of these compounds on sulfhydryl enzymes, are indicating that the reactivity of sulfur-containing protective agents with other cellular constituents exceeds their effects on glutathione. This finding further indicates the necessity of conducting thorough surveys on the actions of radioprotective agents on various biochemical constituents to obtain an indication of their mode of action.

Summary

1. The influence of several radioprotective agents of various types on the reduced glutathione content of rat tissues was studied. Small decreases in some tissues amounting to less than 30% were observed after administration of cyanide, histamine and hydroxylamine. p-Aminopropiophenone, hydroxyacetonitrile, 5-hydroxytryptamine, cysteine, AET, mercaptoethylamine and dimethylammonium dimethyldithiocarbamate caused significant decreases in the reduced glutathione levels of one or more tissues.

2. Measurements of the reduced glutathione content of mouse tissues after administration of various compounds demonstrated that a small reduction in the glutathione levels of various tissues occurred after treatment with the various compounds. However, the effects on glutathione were less pronounced than on sulfhydryl enzymes studied previously suggesting that

TABLE [illegible]

EFFECT OF VARIOUS RADIOPROTECTIVE AGENTS ON THE LEVELS OF OXIDIZED AND REDUCED GLUTATHIONE IN THE TISSUES OF FEMALE RATS

(Animals sacrificed three hours after drug treatment)

Drug	Dose (mgm/kg)	[illegible] Reduced Glutathione					
		Liver		Spleen		[illegible]	
		Ratio Reduced/[illegible]	Reduction	Reduced [illegible]	[illegible]	[illegible]	[illegible]
Control		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible] sulfate	250	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Altitude 30,000 feet for three hours		100 [illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

the reactivity of compounds such as mercaptoethylamine and AET with glutathione cannot be assumed to parallel their reactivity with enzyme systems.

3. Exposure of rats to 900 r of x irradiation caused reduction of the reduced glutathione levels of thymus, erythrocytes and ileum to the extent of 62%, 32% and 17% respectively.

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THE EFFECTS OF IONIZING RADIATIONS ON THE BIOCHEMISTRY OF MAMMALIAN TISSUES

II. The Effects of Various Chemical Compounds on the Nitrogen Mustard-Induced Changes in Enzyme Activities of Certain Tissues of Rats

Bernard F. Miethrink, Ann B. Raymund and Kenneth P. DuBois

This report concerns: The ability of various chemical agents to reduce the nitrogen mustard-induced changes in enzyme activities of the spleen, thymus gland and small intestine of rats. Results of survival studies of animals pretreated with some of these compounds before injection of lethal doses of a nitrogen mustard are also included. This study was performed to compare the effects of these radiomimetic drugs with results obtained in our previous studies on x-irradiated animals.

Immediate or ultimate application of the results: The nitrogen mustards and related alkylating compounds show carcinostatic and several other actions similar to those produced by x-irradiation and are, therefore, called radiomimetic agents. A number of compounds that have radioprotective activity also have the ability to antagonize the toxic effects of the nitrogen mustards. Both cysteine and 2-mercaptoethylamine (MEA) have been used clinically to counteract nausea and vomiting accompanying x-irradiation and methyl bis(2-chlorethyl)amine (HN2) hydrochloride therapy (1,2). A number of earlier studies in this laboratory have illustrated the applicability of adenosine triphosphatase measurements using spleen and thymus glands of irradiated and nitrogen mustard-treated rodents for estimating damage caused to the hematopoietic system. This method has also been used to quantitatively determine the radioprotective effect provided the hematopoietic system by chemical agents. It has also been found in this and other laboratories that x-irradiation causes a dose-dependent decrease in the acetylcholinesterase activity of the small intestine within a few days after exposure; however, results of similar studies using the nitrogen mustards have not been reported. The current study consisted of the application of some of these findings on x-irradiated animals to evaluate the ability of various chemical agents to antagonize the damaging effects of the nitrogen mustards. Investigation of the effect of HN2 on the cholinesterase activity of the small intestine was also carried out. These experiments offered the possibility of obtaining additional information of the mechanisms involved in the protective action provided by various chemical agents and thus aid in our subsequent research on radioprotective compounds.

* * * * * * * *

DuBois et al. (3) have shown that the administration of sublethal and lethal doses of ethyl bis(2-chlorethyl)amine (HN1) and methyl bis(2-chlorethyl)amine (HN2) produce dose-dependent increases in the adenosine triphos-

phatase activity of the hematopoietic tissues of rats and that these increases are maximal at three to four days after injection and are reversible after sublethal doses. The results of these studies indicated that the nitrogen mustards are qualitatively similar to x-ray in their ability to increase the adenosine triphosphatase activity of hematopoietic tissues. Studies of Petersen and Dacie (4) and our recent experiments (5-9) have illustrated the value of adenosine triphosphatase assays on the spleens and thymus glands of rats, pretreated with various chemical agents before radiation, as biological indicators of the radioprotective effect of these compounds. Thus the marked similarities between the effect of x-irradiation and nitrogen mustards on the increase in enzyme activity indicated that adenosine triphosphatase assays might also be applicable as a means of determining to what extent chemical agents protect against nitrogen mustard-induced damage to the hematopoietic tissues. To test this possibility the present study was conducted and several agents known to provide protection against radiation lethality were tested for their ability to reduce the increase in adenosine triphosphatase activity of the spleen and thymus glands caused by HN2.

The effect of nitrogen mustards on the acetylcholinesterase activity of the small intestine has not been studied. However, its clinical use causes nausea and vomiting similar to that resulting from x-irradiation (1,2). Doull and Cummings (10,11) have shown that the acetylcholinesterase activity of the intestine is markedly decreased following total body x-ray and the effect is dose-dependent following exposures as great as 1200 r. Our recent studies (5-9) have illustrated that the degree of modification of the decrease in acetylcholinesterase activity of the small intestine caused by the administration of chemical compounds before exposure to radiation is a valid indication of the protective effect provided this tissue by the compounds. Thus, experiments were undertaken to determine the effect of HN2 on the cholinesterase activity of the small intestine. Results of these studies indicate that HN2 causes a marked decrease in the cholinesterase activity of the small intestine which is not strictly dose dependent, but is maximal at about three days and returns to normal after sublethal doses. The ability of some radioprotective chemical agents to reduce the HN2-induced decrease in acetylcholinesterase activity of the small intestine is also described in this report.

Materials and Methods. Adult female Sprague-Dawley rats were used for these experiments. The animals were maintained in air-conditioned quarters and were fed Rockland rat diet and water ad libitum. The compounds used in this study were administered as neutral aqueous solutions at appropriate times before injection of HN2 hydrochloride. The concentrations of the drugs used in combinations were adjusted to permit intraperitoneal injections of total volumes not exceeding 1.7% of the body weight.

The adenosine triphosphatase activity of the spleens and thymus glands of rats was measured according to the method of DuBois and Potter (12) using 0.5% homogenates of spleen and 1% homogenates of thymus glands. Assays were performed in duplicate using two levels of tissue (0.1 ml. and 0.2 ml. of homogenate). Inorganic phosphorus was determined by the procedure of Fiske and Subbarow (13) and the enzyme activity was expressed as µgm. of phosphorus/mgm. of tissue/15 minutes.

The acetylcholinesterase activity of the small intestine was determined by the manometric method of DuBois and Mangun (14). A portion of the jejunum-ileum was freed from the mesenteric connective tissue and fat and longitudinally dissected to expel the contents. The tissue was then washed twice with distilled water, blotted dry with filter paper, minced and homogenized with Ringer-bicarbonate buffer. Measurements were conducted in duplicate using 50 mgm. of tissue per Warburg vessel. The vessels were gassed with 5% CO_2 and 95% N_2 for five minutes. Carbon dioxide evolution was recorded at five-minute intervals for a period of thirty minutes following a preliminary ten-minute equilibration. Acetylcholinesterase activity was expressed as µl. of CO_2 evolved/50 mgm. of tissue/10 minutes.

Experimental

The effect of various chemical compounds on the adenosine triphosphatase activity of the spleens and thymus glands of rats treated with methyl bis (2-chlorethyl)amine (HN2). DuBois and Petersen (15) have demonstrated that the increase in the adenosine triphosphatase activity of the spleens and thymus glands of rats reached a maximum at approximately three days after x-irradiation or after HN2. Our recent studies (5-9) illustrate that the ability of chemical compounds to reduce the increase in enzyme activity of the spleens and thymus glands following x-ray is a valid means of quantitating the radioprotective effect of these agents on the hematopoietic system. In order to compare the effects of various doses of HN2 on the adenosine triphosphatase activity of hematopoietic tissues with those obtained after various doses of x-ray (5), groups of four to eight female rats were given doses of HN2 ranging from 0.5 mgm./kgm. to 1.75 mgm./kgm. and the enzyme activity of the spleen and thymus glands was determined three days later. The results of these tests, which are presented in Figure 1 are in agreement with the findings of DuBois et al. (13) who showed a dose-dependent relationship between the amount of increase in enzyme activity and the dose of HN2. However, doses of HN2 in excess of 1 mgm./kgm. were required to cause a substantial increase in the enzyme activity of the thymus glands and although sublethal doses cause similar changes in adenosine triphosphatase activity of the spleen the increases produced by HN2 are not as marked as those produced by sublethal exposure to x-irradiation. Therefore, it is more difficult to accurately quantitate the protective effect of chemical agents against the toxicity of HN2 in terms of per cent reduction of the dose than in the case of x-ray (5). Thus chemical protection against HN2 has been considered in this study as the ability of various agents to reduce the increase in adenosine triphosphatase activity. The enzyme changes were expressed in terms of per cent of normal activity.

Several sulfur-containing compounds have been shown to protect rodents against the lethal effect of x-irradiation and nitrogen mustards (16). Therefore, it was of interest to determine the effect of some sulfur-containing radioprotective compounds on the adenosine triphosphatase activity of HN2-treated rats. The results of these studies are presented in Table 1. In all cases the compounds under investigation were given prior to 1.5 mgm./kgm. of HN2, the LD_{50} for rats under the conditions of our experiments. Administration of 1.5 mgm./kgm. of HN2 resulted in an increase in the adenosine triphosphatase activity of the spleen to 188% and of the thymus gland to 20% of normal in

Figure X

The Adenosine Triphosphatase Activity of Spleens and Thymus Glands of Rats After Various Doses of HN2

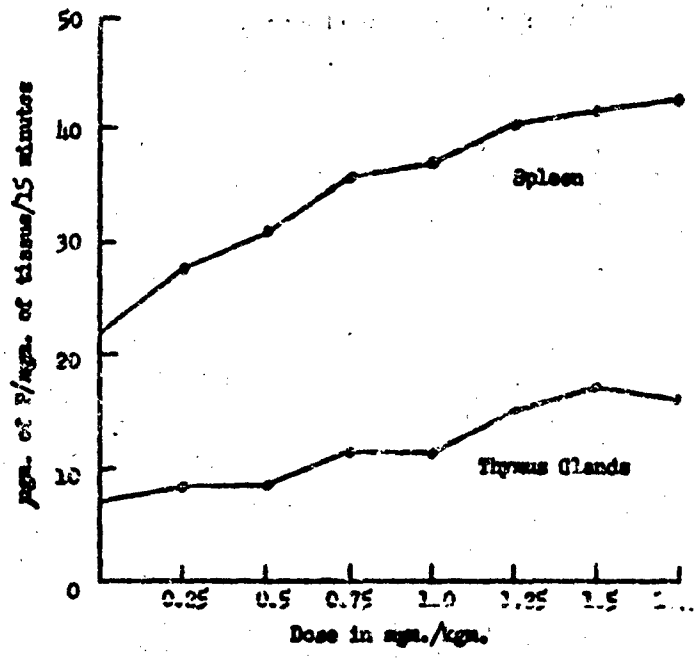

TABLE 1

THE EFFECT OF VARIOUS CHEMICAL COMPOUNDS ON THE ADENOSINE TRIPHOSPHATASE ACTIVITY OF THE SPLEEN AND THYMUS GLANDS OF MICE TREATED WITH

Treatment	Dose in mgm./kgm.	Time of Injection before HN2 (Minutes)	Spleen ATPase		Thymus Gland ATPase	
			Activity	% of Control	Activity	% of Control
Control	.....	..	[illegible]	...	7.3±0.6	...
HN2 (only)	1.5	..	[illegible]	186	14.9±0.8	204
DMDTC	400	5	32.0±3.4	145	15.0±0.9	206
DEDTC	600	5	27.5±0.4	124	13.5±2.2	185
MEA	200	10	36.6±2.6	165	13.5±1.1	185
Cysteine	1,000	15	29.6±1.5	134	9.5±0.2	130
GSH	900	15	36.9±3.4	167	11.6±0.8	159
AET	150	10	42.0±2.8	190	15.9±1.2	209
Cysteine plus DMDTC	1,000 400	15 5	27.3±1.7	123	13.2±1.0	181
GSH plus DMDTC	900 400	15 5	27.3±1.7	123	11.6±1.0	159
Cysteine plus DEDTC	1,000 600	15 5	29.3±1.4	132	15.6±0.6	214
GSH plus DEDTC	900 600	15 5	29.2±1.4	132	12.0±0.6	164
Cysteine plus MEA	1,000 200	15 10	31.5±2.2	143	13.3±0.4	182
Cysteine plus GSH	1,000 900	15 10	28.7±1.3	129	12.0±0.6	164
Hydroxylamine	75	5	[illegible]	177	22.9±0.7	314
Serotonin	20	5	[illegible]	199	17.2±0.2	236

three days. The ability of the dithiocarbamates, which provide significant radioprotective effects in mice (17) and rats (8), to counteract the toxic effects of HN2 has not been previously reported. The ammonium salt of diethyldithiocarbamic acid (DEDTC) reduced the increase in the adenosine triphosphatase activity of the spleen caused by HN2 to 114% of normal but had no effect on the enzyme activity of the thymus glands. The phosphatase activity of the spleen and thymus glands increased to 124% and 185% of normal respectively after pretreatment with the sodium salt of diethyldithiocarbamic acid (DEDTC) given before HN2. Mercaptoethylamine (MEA) and cysteine, which have been used clinically to counteract the adverse effects in HN2 therapy, reduced the increase in enzyme activity of the spleen to 165% and 131% of control respectively. MEA had a slight beneficial effect on the thymus glands and cysteine was the most efficacious of the compounds tested in this study in reducing the increase in the enzyme activity of this tissue. Nine hundred mgm./kgm. of reduced glutathione (GSH) before HN2 caused protection similar to that provided by MEA in the spleen and reduced the increase in adenosine triphosphatase activity of the thymus glands to 159% of normal. This is in agreement with Therkelson (18) who has shown in mice that GSH gives roughly the same protection against HN2 as that provided by MEA. This investigator has also found that AET offers slight, although significant, protection against HN2 lethality in mice but adenosine triphosphatase assays on the spleen and thymus glands of rats do not demonstrate this effect.

Our previous studies (7-9) have demonstrated the additive protective effect provided by certain combinations of chemical compounds against the damaging effects of x-irradiation. Therefore, several combinations of compounds were tested for their ability to reduce the increase in adenosine triphosphatase activity of the spleen and thymus glands of rats treated with HN2. The combination of cysteine or GSH with DEDTC produced additive beneficial effects in the spleen as evidenced by the reduction of the increase in activity to 113% of control in both cases. These combinations did not reduce the effect of HN2 on the thymus glands to a greater extent than cysteine or GSH when administered alone. Combination of cysteine with DEDTC and GSH with DEDTC did not prevent the increase in adenosine triphosphatase activity in the spleens to the extent of DEDTC alone or in the thymus glands to the degree obtained with either cysteine or GSH administered singly. The additive radioprotective effect of cysteine plus MEA has been demonstrated in this laboratory by Petersen and DuBois (4). However, this combination given before HN2 did not reduce the increase in enzyme activities in the hematopoietic tissues studied to the extent of cysteine alone. Beneficial effects of cysteine plus GSH were observed in the spleen but not in the thymus gland of HN2-treated animals.

Since several sulfur-containing radioprotective compounds exhibited the ability to reduce the damaging effects of HN2 on the hematopoietic tissues of rats, it was of interest to determine the effect of a few protective agents that are not of the sulfur-containing type. Hydroxylamine and serotonin were, therefore, included in this study and the results indicate that these compounds fail to reduce the damaging effects of HN2 on the spleen and markedly increase the toxic effect of HN2 on the thymus glands. In both experiments two of five animals died within the three-day period thus indicating an increased toxicity.

It was also noted that the hydroxylamine-induced increase in spleen size that was observed in our recent experiments on radioprotection was not evident three days after HN2.

The effect of various chemical compounds on the acetylcholinesterase activity of the small intestine of HN2-treated rats. In view of the marked similarity of the effects of HN2 and x-irradiation on the adenosine triphosphatase activity of the spleen and thymus glands, it was of interest to determine whether this similarity was also evident in the cholinesterase activity of the small intestine. For this experiment groups of five animals were injected with 0.5 mgm./kgm. of HN2 and the small intestine was assayed for cholinesterase activity at twelve hours and 1, 2, 3, 5 and 7 day intervals. Results of this study are presented in Figure 2. They show that a decrease in the cholinesterase activity of the small intestine is evident in twelve hours and that the decrease is maximal in two to three days with the activity rapidly returning to normal within seven days after a sublethal dose of 0.5 mgm./kgm.

It was also of interest to determine whether the decrease in cholinesterase activity caused by HN2 was dose-dependent as it is in the case of x-irradiation. Thus groups of five rats were injected with various doses of HN2 ranging from 0.25 mgm./kgm. to 1.75 mgm./kgm. The animals were sacrificed three days later and the small intestines assayed for acetylcholinesterase activity. The data shown in Figure 3 illustrate that 0.25 mgm./kgm. of HN2 has little or no effect on the enzyme activity but that 0.5 mgm./kgm. causes a marked decrease similar to that observed with 400 r of x-ray. This decrease, although subject to rather wide variation, is only slightly enhanced by larger doses of HN2. Thus it is evident that although HN2, like x-ray, produced a decrease in acetylcholinesterase activity of the small intestine which is reversible after sublethal doses, it did not produce a dose response curve that is strictly dose-dependent. Hence, it was not possible to accurately determine the quantitative ability of various chemical agents to antagonize the toxic effects of HN2 in the intestine but since several agents (Table 2) reduced the HN2-induced change in acetylcholinesterase activity it was evident that these compounds display a protective effect.

The data in Table 2 indicate that 1.5 mgm./kgm. of HN2 caused a decrease in the cholinesterase activity of the small intestine to 41% of normal. It is interesting to note that DiDTC and DEDTC, which in our previous studies were shown to enhance the damaging effects of x-ray in the intestine, almost entirely prevented the marked decrease in cholinesterase activity caused by HN2. A dose of 200 mgm./kgm. of MEA did not significantly reduce the HN2-induced decrease in enzyme activity. However, administration of cysteine, which has been used effectively against the nausea and vomiting that accompany HN2 therapy, prior to the nitrogen mustard almost completely prevented the decrease in enzyme activity as evidenced by cholinesterase activity levels that were 87% of normal. GSH and AET failed to alter the amount of decrease in cholinesterase activity caused by HN2.

Several combinations of chemical compounds were tested for protection against the decrease in cholinesterase activity following injection of nitrogen mustard. In each case one and sometimes both of the agents given in combination showed indications of marked protection when given alone and, therefore, the

Figure 2

The Effect of HN2 on the Acetylcholinesterase Activity of the Small Intestine of the Rat

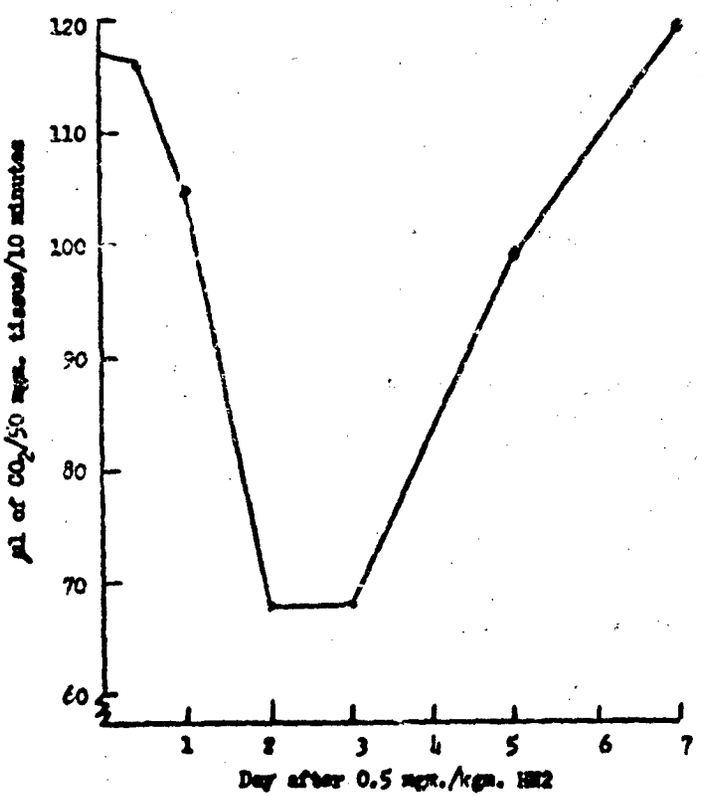

Figure 3

The Acetylcholinesterase Activity of the Small Intestine of the Rat Three Days After Various Doses of HN2

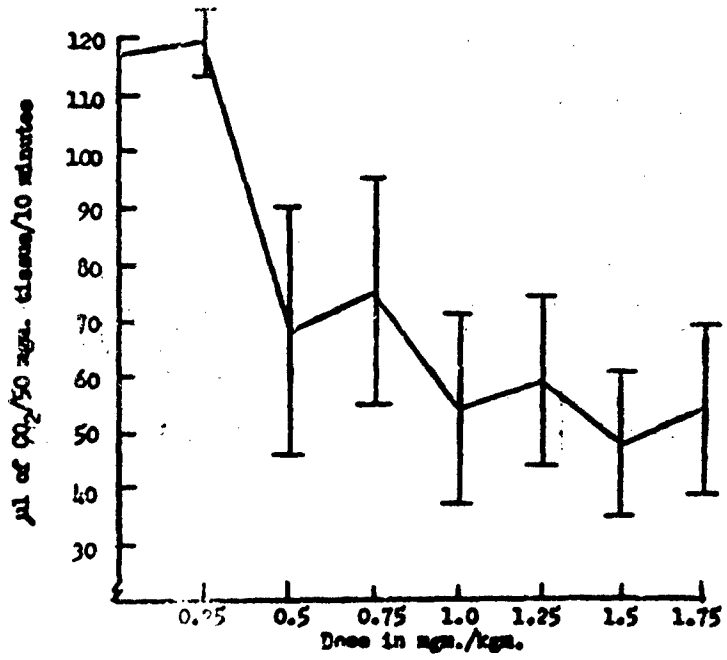

TABLE 2

THE EFFECT [illegible] CHEMICAL COMPOUNDS ON THE ACETYLCHOLINESTERASE ACTIVITY OF THE SMALL INTESTINE OF HN2-TREATED RATS

Treatment	Dose in mgm./kgm.	Time of Injection Before HN2 (Minutes)	Intestinal Cholinesterase	
			Activity	% of Control
Control	.....	..	117±6	...
HN2 (only)	1.5	..	48±13	41
DIDTC	400	5	120±4	102
DEDTC	600	5	112±16	96
MEA	200	10	57±12	49
Cysteine	1,000	15	105±12	90
GSH	[illegible]	15	42±4	36
AET		10	49±5	42
Cysteine plus DIDTC	1,[illegible] 400	15 5	114±19	97
GSH plus DIDTC	900 400	15 5	103±25	88
Cysteine plus DEDTC	1,000 600	15 5	104±19	89
GSH plus DEDTC	900 600	15 5	111±11	95
Cysteine plus MEA	1,000 200	15 10	108±21	92
Cysteine plus GSH	1,000 900	15 10	103±16	88
Hydroxylamine	75	5	39±3	33
Serotonin	20	5	39±5	33

efficacy of these combinations must be determined by the ability to permit survival after lethal doses of HN2.

Hydroxylamine and serotonin, two of the non-sulfur-containing radioprotective compounds, increased the damaging effects of HN2 in the intestine as evidenced by reduction of the acetylcholinesterase activity to 33% of normal. As pointed out earlier in this report 40% of the animals treated with these compounds before HN2 failed to survive the three-day assay time.

The effect of chemical compounds on the 30-day survival of rats after injection of toxic doses of HN2. Results of the study indicated that several chemical agents were capable of reducing the degree of HN2-induced enzyme changes in the hematopoietic tissues and the intestine of rats. To ascertain whether this protective effect could be related to the ultimate survival of the animals some of these agents were administered before injection of doses of HN2 ranging from the LD_{50} dose of 1.5 mgm./kgm. to 5 mgm./kgm. The ability of a compound to protect was then correlated with the increase in the LD_{50} dose of the nitrogen mustard. In preliminary studies 400 mgm./kgm. of DMDTC prevented mortality of 100% and 20% of the animals injected with 2.5 mgm./kgm. and 5 mgm./kgm. of HN2 respectively. Hydroxylamine did not reduce the HN2-induced alterations in the enzyme activities of the tissues studied and pretreatment with this compound resulted in 60% mortality after injection of the LD_{50} dose of nitrogen mustard. Further studies are currently in progress to more exactly determine the ability of DMDTC to counteract the toxicity of HN2 and to correlate the reduction in tissue damage provided by other sulfur-containing compounds with survival after increased doses of HN2.

Discussion

The results presented in this report demonstrate the application of enzyme assays on tissues sensitive to nitrogen mustard damage as a means of evaluating the protective effects provided by chemical agents. The findings illustrate the possibility that certain combinations of chemical agents may provide additive protective effects; however, quantitative evaluation of the beneficial effects requires survival data because HN2 does not cause strictly dose-dependent enzyme changes in the thymus glands and intestine. The sulfur-containing compounds employed in this study that reduced the alterations in enzyme activity to the greatest extent were cysteine, DMDTC, and TMDTC.

The marked degree of reduction of the HN2-induced increase in enzyme activity produced by cysteine as compared to that caused by MEA may be proportional to the relative toxicity of the two compounds. This observation is in agreement with the results obtained by Salerno and Friedell (19) who found that the magnitude of protection of these compounds against the toxicity of HN2 was directly proportional to the molar dosage. Goldenthal et al. (16) have clearly demonstrated with labeled HN2 that this nitrogen mustard combines with cysteine in vivo. Mice pretreated with cysteine before HN2 injection excreted a reaction product of the two compounds in the urine and there was a partial breakdown of the reaction product of HN2 and cysteine when it was administered to mice. Therefore, it appears that the protection in animals pretreated with cysteine and then given HN2 is at least partially due to chemical inactivation in vivo. These investigators also found that

sodium nitrate, which acts in x-ray protection presumably by producing a methemoglobinemia, does not reduce the toxicity of HN2. One would not expect agents such as hydroxylamine and serotonin to give protection against HN2 since as stated by van Rekkum (20), biological amines may exert their radioprotective effect by impairment of circulation and subsequent reduction in tissue oxygen tension. Oxygen tension influences radiation injury but not the action of HN2 (18).

It was interesting to note that DMDTC and DEDTC, which have been shown in this laboratory (1) to enhance the damaging effects of x-irradiation in the intestine, almost completely reduced the damaging effects of 1.5 mgm./kgm. of HN2 in this tissue. Recent studies of Therkelsen (18) have suggested that the main cause of death in mice after administration of a lethal dose of HN2 is primarily due to intestinal damage. Thus the ability of the dithiocarbamates to reduce the damaging effects of this nitrogen mustard on the small intestine may partially explain the protective effect of these compounds against normally lethal doses of HN2. This investigator has also shown that AET provided slight protection against HN2-induced mortality in mice. Results of this study show, however, that AET is not capable of reducing the damage caused by this nitrogen mustard in the tissues under investigation when the enzyme assays are used as a measure of protection.

As was pointed out earlier in this report it is difficult to ascertain the beneficial effects of combinations of chemical agents against the damaging effects of HN2. Therefore, further experiments including 30-day survival studies, are currently in progress to more accurately determine the efficacy of the treatment with combinations of various sulfur-containing chemical compounds.

Summary

1. Doses of HN2 ranging from 0.25 mgm./kgm. to 1.75 mgm./kgm. were administered to rats and the spleens and thymus glands of these animals were assayed for adenosine triphosphatase activity three days later. Results of this study showed that the increase in enzyme activity of the spleen caused by HN2 was dose-dependent within this dose range but that the nitrogen mustard caused significant increases in the adenosine triphosphatase activity of the thymus glands only after doses in excess of 1 mgm./kgm.

2. Several chemical compounds and combinations of these compounds, known to protect against the damaging effects of x-irradiation, were tested for their ability to reduce the HN2-induced increases in the adenosine triphosphatase activity of the spleen and thymus glands. DMDTC, DEDTC, cysteine and combinations that included these agents were capable of markedly reducing the increase in enzyme activity of the spleen caused by HN2. MEA and GSH were slightly beneficial while AET, hydroxylamine and serotonin failed to reduce the increase in adenosine triphosphatase activity produced in the spleen by the nitrogen mustard. Cysteine was the most effective of the agents studied in reducing the increase in adenosine triphosphatase activity of the thymus glands. DMDTC, AET and

the combination of cysteine plus DEDTC had no beneficial effects while hydroxylamine and serotonin potentiated the effect of HN2 on this tissue.

3. Measurements of the effect of 0.5 mgm./kgm. of HN2 on the acetylcholinesterase activity of the small intestine indicated that this nitrogen mustard caused a decrease in activity that was evident at twelve hours after injection. The enzyme change reached a maximum at two to three days and returned to normal after seven days. A dose of 0.25 mgm./kgm. did not cause any measurable effect on the intestinal cholinesterase activity and doses in excess of 0.5 mgm./kgm. did not produce further dose-dependent decreases.

4. Injection of DMDTC, DEDTC, cysteine or combinations of compounds that included these agents before HN2 markedly reduced the decrease in cholinesterase activity caused by the nitrogen mustard. MEA appeared to have a slight beneficial effect, while GSH, AET and the two non-sulfur-containing compounds, hydroxylamine and serotonin, did not prevent this decrease.

5. DMDTC protected 100% and 20% of the animals against the lethal effects of 2.5 mgm./kgm. and 5 mgm./kgm. of HN2 respectively. Hydroxylamine failed to alter the effect of the LD_{50} dose of 1.5 mgm./kgm. of the nitrogen mustard.

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THE EFFECTS OF IONIZING RADIATIONS ON THE BIOCHEMISTRY OF MAMMALIAN TISSUES

III. Studies on the Toxicity of Rare Earth Compounds and Their Influence on Radiation Lethality

Bernard E. Hiethrink and Kenneth P. DuBois

This report concerns: A continuation of a recently initiated study to obtain information on the toxicity of rare earth compounds. Special consideration has been placed upon those agents which would be expected to contaminate the atmosphere as a result of a reactor accident or a nuclear detonation. Results of experiments to determine the possible additive or synergistic effect of simultaneous exposure to rare earth oxides and ionizing radiations are also included.

Immediate or ultimate application of the results: The possible hazards associated with an explosive reactor accident has generated considerable interest in the actions of rare earth metals. However, until recently the bulk of the research on rare earths has been centered on the radiotoxicity, tissue distribution and rate of excretion of rare earth isotopes. There is only limited information available on the chemical toxicity of these compounds and the effects of simultaneous exposure to ionizing radiations and rare earth elements. Thus, a program was recently undertaken to obtain further information on the acute toxicity of rare earth compounds and the effects of exposure of rats to rare earth oxides plus ionizing radiation. Information of this type may be of value in determining the health hazards associated with industrial use of the rare earth metals and the possible danger arising from fission product contamination resulting from extensive atomic energy developments.

* * * * * * * * *

Various approaches to the problem of determining the toxicity and mode of action of rare earth metals have been undertaken. In this connection Hartwig et al. (1) have shown that placing yttrium nitrate or lanthanum nitrate in wounds caused healing to be retarded for several weeks. Melville et al. (2) reported similar findings with didymium nitrate. Maxwell and Hischoff (3) found that yttrium injected intravenously will cause protein precipitation and several investigators (1-5) have reported the formation of large abscesses following subcutaneous or intraperitoneal injection of various rare earth salts. Histopathologic studies by Steffee (5) have shown that intraperitoneal administration of the stable rare earths including cerium, lanthanum, yttrium, praseodymium and neodymium at relatively high dosage levels caused an acute peritoneal reaction which developed into a true granulomatous peritonitis and occasionally resulted in focal hepatic necrosis. However, the available information on the acute toxicity of rare earths is limited. The recent reports of Cochran et al. (6), Craca et al. (4) and of Kylar and Cress (7) contain most of the published measurements. Since much of the toxicity work on rare earths is of a preliminary nature and did not include several of the rare earth metals, it was

evident that further research on the toxicity of these chemical agents was needed.

The recent finding (1,2) of synergistic toxicity resulting from the simultaneous exposure of rats to dysprosium nitrate or yttrium nitrate plus radiation indicates the importance of conducting thorough studies on the possible combined actions of other rare earths and ionizing radiation. In our previous study (8) preliminary experiments were done in which rats were given sublethal doses of the oxides of several rare earth metals plus a lethal dose of x-ray. Praseodymium oxide and gadolinium oxide shortened the survival time of irradiated animals to some extent but the other compounds tested had no detectable effect on mortality or survival time. However, since a lethal dose of x-irradiation was employed it was difficult to detect a synergistic effect. Therefore, additional experiments were conducted in which a dose of x-ray that did not cause death of all of the animals was given after injection of 1000 mgm./kgm. of the rare earth oxides.

The present report contains additional data on the toxicity of the oxides and nitrates of several rare earth metals. The combined effect of rare earth metals and 775 r of x-irradiation on the mortality and weight changes of rats are included.

Materials and Methods. Adult female Sprague-Dawley rats were used in these studies. The animals were housed in air-conditioned quarters and fed Rockland rat diet and water ad libitum. The oxides of rare earth metals were obtained from the St. Kloi Corporation, Cincinnati, Ohio and the nitrates of gadolinium, ytterbium, didymium, samarium, praseodymium and neodymium from the K and K Laboratories, Jamaica, New York. The remaining nitrate salts were obtained from the Lindsay Chemical Company, West Chicago, Illinois. The nitrates were administered intraperitoneally as unneutralized aqueous solutions. The oxides were suspended by homogenization in 0.2% carboxymethylcellulose and given by either the oral or intraperitoneal route. After injection of the compounds the animals were observed for a period of thirty days. The mortality data presented in this report are based on this observation period.

X-irradiation was administered as a single, whole-body exposure with a G.E. Maximar therapy unit employing the following radiation factors: 250 KVP, 15 ma, 0.25 mm. Cu and 1 mm. Al added filtration. The target-animal distance was 73 cm. and the dose rate was 37 r to 39 r per minute as measured in air with a Victoreen ionization chamber.

Experimental

Acute intraperitoneal and oral toxicity of rare earth oxides to female rats. Results of our previous study (8) on the intraperitoneal and oral toxicity of rare earth oxides show that occasional deaths occurred in some of the groups of animals receiving up to 800 mgm./kgm. intraperitoneally or 200 mgm./kgm. of the oxides orally. Additional experiments were, therefore, undertaken to determine if these random deaths were a reflection of the toxicity of the oxides. Thus female rats were given intraperitoneal and oral doses of 1000 mgm./kgm. of the rare earth oxides and observed for a 30-day period. The results of these experiments are shown in Table 1.

TABLE 1

MORTALITY OF RATS GIVEN 1000 MGM./KGM. OF RARE EARTH OXIDES BY THE ORAL AND INTRAPERITONEAL ROUTES

Compound	Route of Administration	Mortality	% Mortality
Neodymium oxide	Intraperitoneal Oral	1/5 0/5	20 0
Praseodymium oxide	Intraperitoneal Oral	0/5 0/5	0 0
Samarium oxide	Intraperitoneal Oral	0/5 0/5	0 0
Gadolinium oxide	Intraperitoneal Oral	1/5 0/5	20 0
Ytterbium oxide	Intraperitoneal Oral	0/5 0/5	0 0
Dysprosium oxide	Intraperitoneal Oral	0/5 0/5	0 0
Europium oxide	Intraperitoneal Oral	0/5 0/5	0 0
Holmium oxide	Intraperitoneal Oral	0/5 0/5	0 0
Erbium oxide	Intraperitoneal Oral	2/5 0/5	40 0
Ceric oxide	Intraperitoneal Oral	0/5 1/5	0 20
Terbium oxide	Intraperitoneal Oral	0/5 1/5	0 20

In every case most of the animals were able to tolerate 1000 mgm./kgm. of the rare earth oxides and the LD_{50} values are, therefore, in excess of this dosage level for all of the compounds. A few deaths did occur during the observation period. One of a group of five rats that received neodymium oxide intraperitoneally died as was the case after 800 mgm./kgm. but the group of three animals given 1600 mgm./kgm. survived for thirty days (8). Mortality of one of the animals occurred seven days after gadolinium oxide and 40% of the rats receiving erbium oxide intraperitoneally failed to survive. Thus it appears that, with the possible exception of erbium, rats are able to tolerate intraperitoneal doses of rare earth oxides considerably in excess of 1000 mgm./kgm.

Studies were also undertaken to compare the toxicity of the rare earth oxides given by the intraperitoneal and oral routes. Previous results showed that rats are able to tolerate 200 mgm./kgm. of all the compounds given orally. Hence suspensions of the rare earth oxides were given orally at the dosage level of 1000 mgm./kgm. [illegible] dose caused the death of one rat in each of the groups of animals given [illegible] oxide and terbium oxide. The remainder of the animals that received 1000 mgm./kgm. of the oxides of the various rare earth metals survived the 30-day test period. It was interesting to note that oral administration of the oxides of praseodymium, gadolinium, cerium and terbium appears to inhibit the normal weight increase. The animals did not lose weight but remained at a steady level for approximately fifteen days following the administration of the oxide. Further tests will be performed to more accurately determine the effect of these oxides on the weight gain.

Acute intraperitoneal toxicity of rare earth nitrates to female rats. Preliminary studies (8) on six nitrate salts of rare earth metals indicated that the LD_{50} values for neodymium, praseodymium, samarium, gadolinium, ytterbium, and didymium were between 200 mgm./kgm. and 400 mgm./kgm. Therefore, additional toxicity studies to obtain a more exact estimate of the LD_{50} values of these agents were performed. The data summarized in Table 2 show the results of these experiments. Recently we obtained the nitrate salts of four other rare earth metals. The results of initial toxicity tests on three of these compounds, namely cerium, holmium, and europium nitrates, are also shown in Table 2. The determination of the LD_{50} of the fourth rare earth, terbium nitrate, is currently in progress.

These tests indicate that the nitrate salts of neodymium, praseodymium, samarium, gadolinium, ytterbium and didymium exhibit similar toxicities. The LD_{50} of all these agents is approximately 275 mgm./kgm. This similarity in toxicity is in agreement with studies done by Draca et al. (4) who found that when rats were given intraperitoneal injections of cerium chloride, praseodymium chloride and neodymium chloride, LD_{50} values of 353, 358 and 348 mgm./kgm. were obtained. Initial studies indicate that europium nitrate and erbium nitrate are more toxic since these compounds caused death of 80% of the animals given a dose of 250 mgm./kgm. A similar dose of holmium nitrate produced mortality of two of a group of five rats suggesting that its LD_{50} is also near 275 mgm./kgm. Further studies to obtain a more accurate estimate of the LD_{50} values of europium, holmium and erbium nitrates are currently in progress.

Influence of rare earth oxides on the survival time and weight changes in rats exposed to [illegible] r of x-ray. In our initial experiments (8) on the influence of rare earth oxides on the degree of radiation injury, animals were

TABLE 2

ACUTE INTRAPERITONEAL TOXICITY OF RARE EARTH NITRATES TO FEMALE RATS

Compound	Dose (mgm./kgm.)	Mortality	% Mortality
Neodymium nitrate	400 300 250	5/5 3/5 2/5	100 60 40
Praseodymium nitrate	300 250	4/5 1/5	80 20
Samarium nitrate	300 250	3/5 1/5	60 20
Gadolinium nitrate	300 250	4/5 2/5	80 40
Ytterbium nitrate	300 250	4/5 2/5	80 40
Didymium nitrate	300 250	4/5 2/5	80 40
Europium nitrate	250	4/5	80
Holmium nitrate	250	2/5	40
Erbium nitrate	250	4/5	80

given intraperitoneal doses of 600 mgm./kgm. of the compound 10 to 15 minutes before an ordinarily lethal amount of x-irradiation. Since a lethal dose of x-ray was employed, it was difficult to determine whether the oxides produced a synergistic effect with radiation as was found when didymium nitrate or ytterbium nitrate were injected before x-ray (1,2). It was, therefore, of interest to determine the effect of rare earth oxides on the survival time and weight changes in rats exposed to doses of x-ray that did not produce 100% mortality. For this study groups of five animals were given intraperitoneal injections of 1000 mgm./kgm. of the rare earth oxides fifteen minutes prior to 775 r of x-irradiation. As shown in Table 1 this dose of rare earth oxide produced only an occasional death of an animal. Two of the ten control rats exposed to 775 r survived the 30-day test period.

Figure 1 shows that the oxides of neodymium and praseodymium did not appreciably affect the survival time or mortality of rats exposed to 775 r. Samarium oxide shows evidence of producing a slight degree of radioprotection as evidenced by the increased ST_{50} and survival of 60% of the animals. The data in Figure 2 show that gadolinium and dysprosium had no influence on the survival of irradiated rats. However, ytterbium oxide permitted survival of two of the five animals given 775 r. As shown in Figure 3 europium oxide decreased the survival time slightly and caused death of the five rats exposed to 775 r. Holmium oxide and erbium oxide caused mortality of two of the five animals given the compounds without radiation (Table 1) and when given together with radiation 60% mortality resulted. Cerium oxide did not influence the survival time or mortality of irradiated rats but terbium significantly increased the ST_{50} and permitted 40% survival (Figure 4).

The rare earth oxides did not produce a significant effect on the weight loss of animals following x-irradiation. In no case was the loss of body weight greater with radiation plus the rare earth than with radiation alone. However, it was noted in cases where there was 40% or greater survival at thirty days after x-ray that the initial decrease in weight following irradiation with rare earth oxides was not as marked as with radiation alone. This effect is shown in Figure 5 where it may be seen that the oxides of praseodymium, holmium and terbium, which provided 60%, 40% and 40% survival respectively, substantially reduced the initial weight loss normally associated with whole body x-irradiation. This effect was evident at the third day and continued until about the fourteenth day. The later effects shown in Figure 5 cannot be definitely attributed to the metals because after death of part of the animals in a small group the average weight of the remaining animals has less significance.

Discussion

The present investigation consisted of additional experiments to ascertain the chemical toxicity of rare earth metals, the possible hazards associated with the industrial use of these compounds and the influence of simultaneous exposure to rare earth metals and ionizing radiation. Results of toxicity studies indicated that LD_{50} values of the rare earth oxides are in excess of 1000 mgm./kgm. when these compounds are administered by the intraperitoneal or oral routes. The LD_{50} values of most of the water-soluble rare earth nitrates under investigation appears to be approximately 275 mgm./kgm. although initial results indicate that

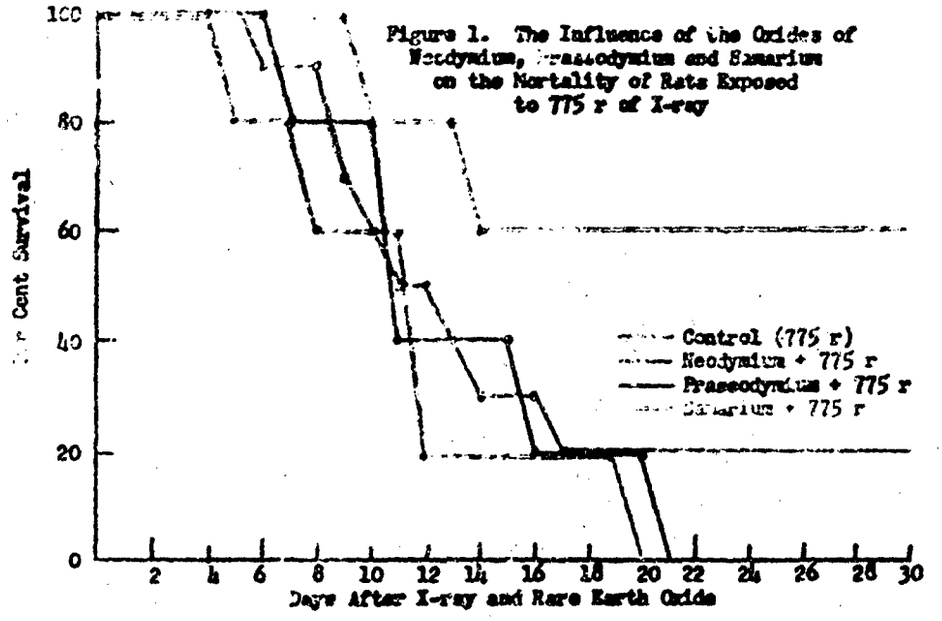


Figure 1. The Influence of the Oxides of Neodymium, Praseodymium and Samarium on the Mortality of Rats Exposed to 775 r of X-ray

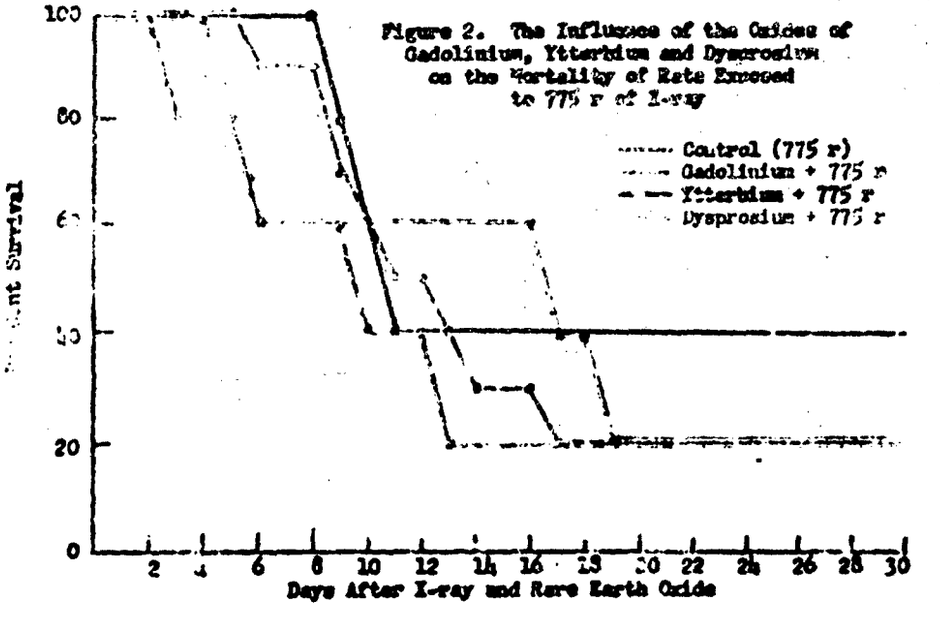


Figure 2. The Influence of the Oxides of Gadolinium, Ytterbium and Dysprosium on the Mortality of Rats Exposed to 775 r of X-ray

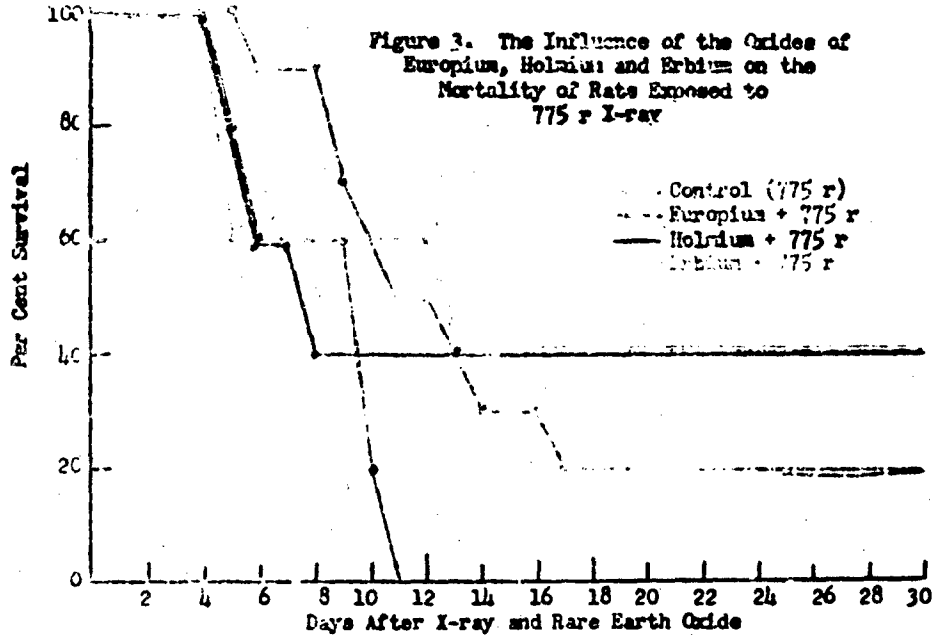


Figure 3. The Influence of the Oxides of Europium, Holmium and Erbium on the Mortality of Rats Exposed to 775 r X-ray

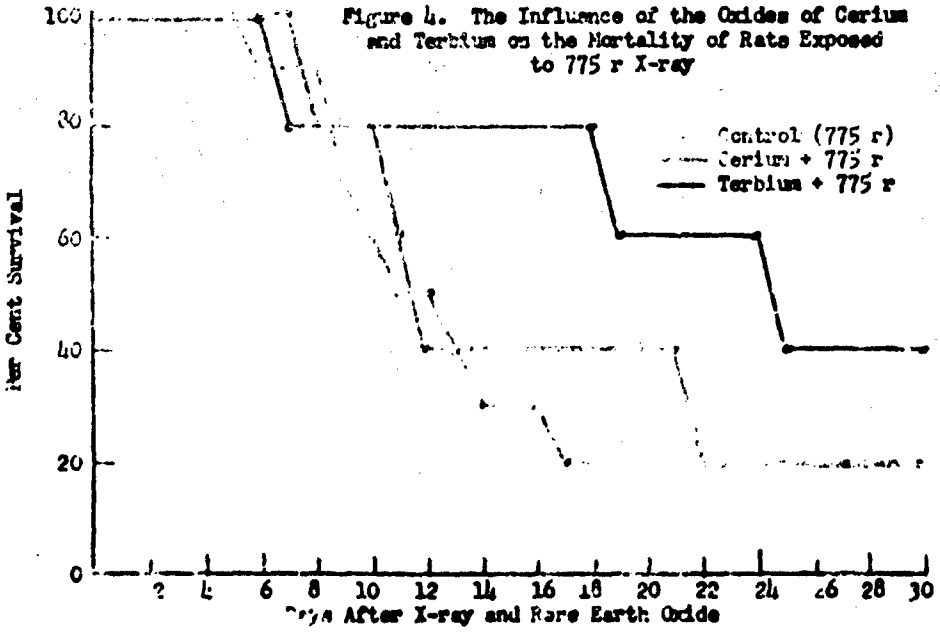
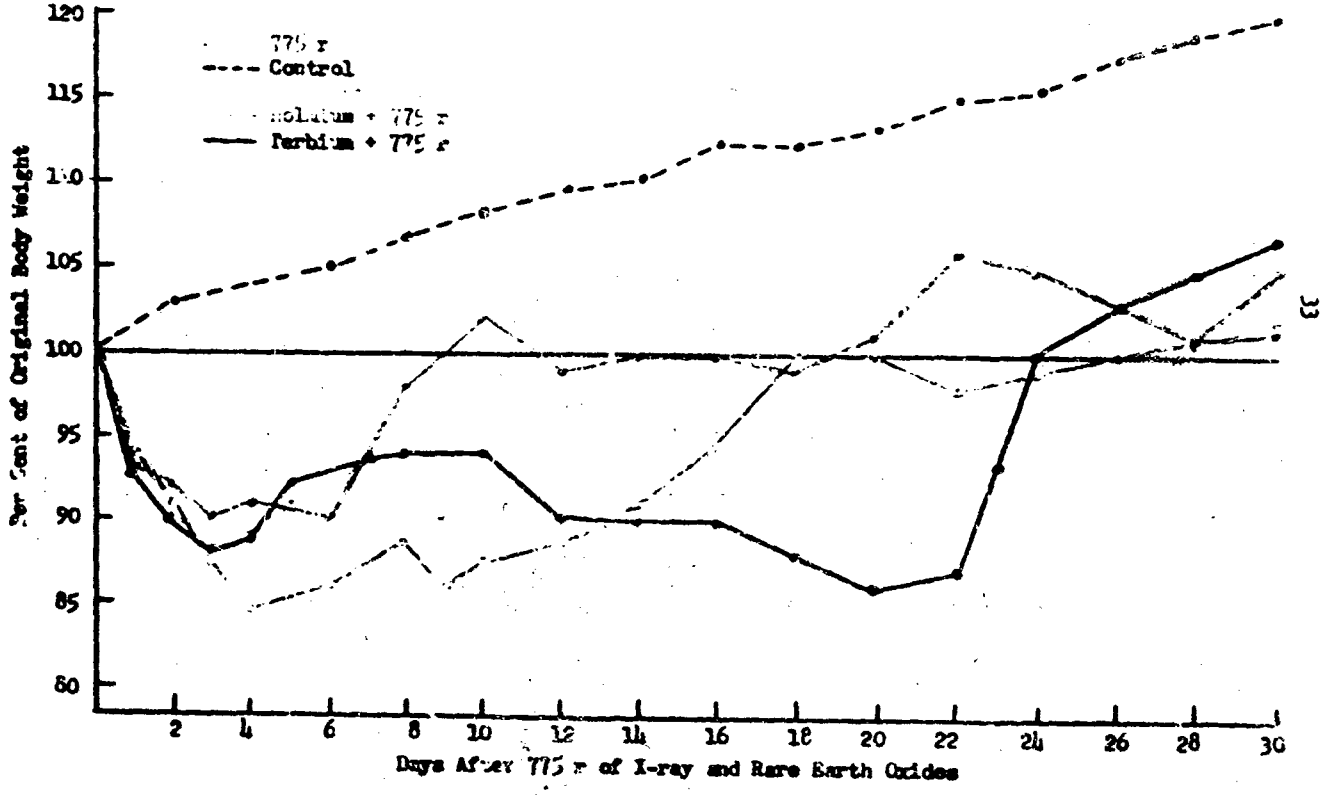


Figure 4. The Influence of the Oxides of Cerium and Terbium on the Mortality of Rats Exposed to 775 r X-ray

Figure 5

Effect of Some Rare Earth Oxides on the Weight Loss of Rats After 775 r of Total Body X-irradiation



europium and erbium nitrate are more toxic than others included in this investigation.

There has been a limited amount of research concerned with the mode of action of the rare earth salts. Cochran et al. (6) in this laboratory have shown by in vitro studies that potassium columbate and the chloride salts of columbium, lanthanum, strontium, yttrium and zirconium inhibited the adenosine triphosphatase activity of mouse liver. These investigators also found that lanthanum chloride was able to replace fluorinum and that strontium chloride was partially effective in replacing this ion in the succinic dehydrogenase system. Other studies have shown that 95% of injected holmium remains unabsorbed at the site of injection four days following administration. However, when this element was administered as the citrate complex only 5% remained unabsorbed. Thus, the studies of Graca et al. (4) show that the citrate complex enhances the absorption and toxicity of the rare earth metals in mice and guinea pigs.

Our preliminary experiments indicate that the toxicity of rare earth oxides is not appreciably affected by x-irradiation when these compounds are administered intraperitoneally at a dose level of 1000 mgm./kgm. fifteen minutes before 775 r. Studies done by Hartwig et al. (1) and by Melville et al. (2) have shown that the subjection of rats receiving intraperitoneal injections of ytterbium nitrate or didymium nitrate to ionizing radiation results in a statistically significant increase in mortality as compared with animals given either radiation or intraperitoneal injections of these compounds alone. In view of the limited research on the rare earth metals, the increasing importance of these compounds in industry and the realization of the possible dangers associated with radioactive accidents these compounds should be subjected to a wide spectrum of tests and their potential hazard more completely evaluated.

Summary

1. Toxicity studies have been conducted on the oxides of neodymium, praseodymium, samarium, gadolinium, ytterbium, dysprosium, europium, holmium, erbium, cerium and terbium. The results of these experiments indicated that the LD_{50} values for these rare earth oxides exceeds 1000 mgm./kgm. when they are given orally or intraperitoneally to rats.

2. The approximate LD_{50} values of the nitrates of neodymium, praseodymium, samarium, gadolinium, ytterbium and didymium have been determined. The LD_{50} values for all of these compounds are between 250 and 300 mgm./kgm. Preliminary toxicity studies indicate that holmium nitrate will have a similar LD_{50} value while europium nitrate and erbium nitrate appear to be more toxic compounds.

3. Measurements of the influence of 1000 mgm./kgm. of various rare earth oxides on the mortality and survival time of rats given 775 r of x-ray simultaneously indicated that only europium oxide decreased survival time. The toxicity of the other oxides tested did not appear to be significantly increased by x-irradiation. The oxides of samarium, holmium and terbium decreased the initial radiation-induced loss of body weight in rats exposed to 775 r.

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THE EFFECTS OF IONIZING RADIATIONS ON THE BIOCHEMISTRY OF MAMMALIAN TISSUES

IV. Effects of Derivatives of Dithiocarbamic Acid on the Oxidation of Alpha-Keto acids

Kenneth P. DuBois, Ann B. Raymund and David R. Seidel

This report concerns: Recent studies on the mechanism responsible for the radioprotective action and mammalian toxicity of derivatives of dithiocarbamic acid. The present study has been concerned with the inhibitory action of dithiocarbamates on the sulfhydryl enzyme systems which catalyze the oxidation of pyruvate and alpha-ketoglutarate.

Immediate or ultimate application of the results: A considerable amount of attention has been given to studies on the mode of action of radioprotective agents in this laboratory during the past two years. The objective of this program is to extend knowledge of the manner in which certain chemicals act to reduce the injurious effects of ionizing radiations. It is anticipated that research of this type will aid in the development of new protective agents and in the acquisition of further information on the mechanism of radiation injury. A great deal of interest has been exhibited in the possibility that the interaction of sulfur-containing radioprotective agents with sulfhydryl enzymes is involved in the protective action of these compounds through the formation of labile mixed disulfide linkages. This type of mechanism could operate to shield reactive groups of enzyme molecules from the oxidizing radicals formed by exposure of tissues to ionizing radiations. This explanation assumes that radiation inactivates sulfhydryl enzymes in vivo. Thus far, however, conclusive data in support of this supposition is lacking and further studies are, therefore, necessary for a resolution of this aspect of the mechanism of radiation injury. It seems likely that the toxic effects of the dithiocarbamates are the result of inhibition of the enzymes included in the present investigation as well as other enzymes containing sulfhydryl groups. Since the radioprotective activity of all sulfur-containing compounds is limited by their toxicity, further information on the mechanism of their toxic actions is needed. If toxicity and radioprotection activity of these compounds involves the same mechanism, it is clear that no increase in radioprotective activity can be achieved by reduction of toxicity. This is an important practical problem to which the type of experiments described in this report may contribute information.

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Within recent months studies were undertaken (1,2) in this laboratory to obtain information on the biochemical effects of derivatives of dithiocarbamic acid on enzyme systems of mammalian tissues. Our attention was attracted to this group of compounds because they appeared to have some advantages over the thioronine derivatives for mechanism studies. The latter group of protective agents undergo a number of chemical changes with the resultant formation

of various metabolic derivatives. Thus the biological activity of the thiuronium derivatives on in vitro systems might differ rather prominently from the effects that occur in the intact animal. The dithiocarbamates are simpler compounds whose in vitro actions might be expected to more closely approximate in vivo effects and, indeed the results obtained in our studies to date have substantiated this supposition.

The radioprotective activity of derivatives of dithiocarbamic acid has been well-established by the previous studies of Bacq et al. (3,4), van Bekkum (5) and van Bekkum and Cohen (6). Some possible explanations for the radioprotective action of these compounds (7) have been published but the ideas were not tested experimentally. Pihl and Eldjarn (8) have recently advanced the interesting theory that protective agents containing sulfhydryl groups form mixed disulfides with cellular constituents and thereby temporarily shield cellular sulfhydryl groups. Previous studies in this laboratory (9) on the mode of action of aminoethylisothiourea (AET) demonstrated that this radioprotective agent reacts with several sulfhydryl enzymes and some preliminary experiments (10) on dithiocarbamates demonstrated that they also have the capability of reacting with sulfhydryl groups of some enzyme systems. In view of these preliminary findings a systematic study of the actions of derivatives of dithiocarbamic acid seemed worthwhile.

The initial phase of our study on the mechanism of the toxic and radioprotective effects of dithiocarbamic acid derivatives consisted of measurement of the blood levels of injected dithiocarbamates. This was accomplished by modification of a method originally developed for copper analysis (11) which depends upon the ability of dithiocarbamates to form a colored complex with copper. Measurements of the blood levels of injected dithiocarbamates showed that these compounds were rapidly absorbed as evidenced by the occurrence of the highest blood levels at five minutes after intraperitoneal injection. At one hour after intraperitoneal administration nearly all of the drug had disappeared from the blood. However, since the inhibitory action of these compounds on various enzymes (1,2) persists for several hours, it is clear that at least that fraction of the total dose which combines with tissue constituents remains in the tissues considerably longer than would be suggested by the blood levels.

A study of the reactivity of dithiocarbamates with enzymes of intermediary carbohydrate metabolism was initiated by measurements of their influence on the oxidation of pyruvate and fumarate by tissue homogenates. When this combination of substrates is used the entire citric acid cycle is operative. If no inhibition is observed consideration of the individual enzymes in the cycle is unnecessary but inhibition of this system indicates that further studies should be conducted to locate the site of the inhibitory effect. The sodium and dimethylammonium salts of dithiocarbamic acid inhibited oxygen consumption, pyruvate utilization and citrate synthesis by tissue homogenates when pyruvate plus fumarate were used as substrates. However, bis(dimethyldithiocarbamyl) disulfide was equal to the dimethylammonium salt of dimethyldithiocarbamate in its inhibitory potency in vitro although it has no protective activity against radiation lethality. These in vitro measurements were followed by a study of the effects of various doses of the dimethylammonium salt of dimethyldithiocarbamate on the oxidation of pyruvate plus fumarate by rat liver and kidney. The animals were sacrificed at various times after

administration of several doses of the compound. The results of these studies clearly demonstrated that doses of dimethylammonium dimethyldithiocarbamate ranging from 166 to 1,000 mgm./kgm. caused marked inhibition of pyruvate utilization and citrate formation by the livers of rats when the animals were sacrificed thirty minutes after injection of the compound. Oxygen consumption by the liver was unaffected. The kidneys of these animals exhibited marked depression of oxygen consumption, pyruvate utilization and citrate formation. Measurements of the duration of the inhibitory action of this compound revealed that rapid reversal of the inhibitory effect occurs. Within five hours after doses as high as 500 and 750 mgm./kgm. of the dimethylammonium salt of dimethyldithiocarbamic acid, the activity of the enzymes of the citric acid cycle had returned to normal. Measurements of the influence of bis(dimethyldithiocarbamyl) disulfide on oxygen consumption, pyruvate utilization and citrate synthesis demonstrated that the maximum tolerated dose of this compound had no appreciable effect in vivo in contrast to its strong inhibitory action in vitro. This finding was of particular interest because this compound has no radioprotective activity.

The present investigation consisted of measurements of the effects of dimethylammonium dimethyldithiocarbamate and bis(dimethyldithiocarbamyl) disulfide on the oxidation of alpha-keto acids. The oxidation of alpha-ketoglutarate to succinate and the oxidation of pyruvate in the presence of malonate were studied. Both of these reactions are catalyzed by sulfhydryl enzymes. The results of these studies demonstrated that the dimethylammonium salt of dithiocarbamic acid is an effective inhibitor of the oxidation of alpha-keto acids in vitro and in vivo. Bis(dimethyldithiocarbamyl) disulfide also inhibited the reactions but to a lesser extent than the dithiocarbamic acid salts.

Materials and Methods. Young adult, male rats (200 to 250 grams), adult male Carworth mice and young male guinea pigs (150 to 300 grams) were used for these experiments. Dimethylammonium dimethyldithiocarbamate was dissolved in 0.1 M phosphate buffer (pH 7.4) and injected intraperitoneally. The low solubility of bis(dimethyldithiocarbamyl) disulfide necessitated the use of propylene glycol solutions.

The oxidation of pyruvate was measured using the test system of Pardee and Potter (12) with a final concentration of 0.0035 M pyruvate. Malonate was added to this system at a final concentration of 0.004 M. Under these conditions the two-carbon fragment resulting from the decarboxylation of pyruvate by liver condenses with another two-carbon fragment resulting in quantitative formation of acetoacetate (13). Thus the oxidation of two moles of pyruvate by liver in this system results in the consumption of one mole of oxygen and the formation of one mole of acetoacetate. The oxidation of alpha-ketoglutarate was measured by the procedure of Ackermann (14). Measurements of oxygen consumption and the utilization of alpha-keto acids were made. Pyruvate and alpha-ketoglutarate were analyzed by the procedure of Friedemann and Haugen (15).

Experimental

Inhibitory effect of derivatives of dithiocarbamic acid on the oxidation of alpha-keto acids in vitro. In the initial studies on the mode of action of dithiocarbamic acid derivatives in this laboratory (1) in vitro experiments were

conducted to ascertain the influence of these compounds on the oxidation of pyruvate plus fumarate by rat liver and kidney homogenates. It was found that the sodium, diethylammonium and dimethylammonium salts of dithiocarbamic acid and bis(dimethyldithiocarbamyl) disulfide caused 50% inhibition of the oxidation of these substrates by liver homogenates at final concentrations ranging from 1×10^{-4} to 5×10^{-4} M on the basis of oxygen consumption. On the basis of chemical analysis of the amount of pyruvate utilized the concentrations required to produce 50% inhibition of the citric acid cycle were essentially the same except that bis(dimethyldithiocarbamyl) disulfide produced 50% inhibition of pyruvate utilization at a concentration of 2.5×10^{-6} M. Analysis of the amount of citrate synthesized by this system as a basis for estimating the inhibitory potency of the compounds also showed that the inhibitory potency of the disulfide was more pronounced than that of the salts of dithiocarbamic acid and the dimethylammonium salt had greater potency than the other salts as an inhibitor in vitro. Kidney homogenates were more susceptible to inhibition by these compounds than was liver. Thus, on the basis of inhibition of oxygen consumption, the sodium and diethylammonium salts produced 50% inhibition at concentrations of about 4×10^{-5} M. The dimethylammonium salt and the disulfide were more active inhibitors as evidenced by 50% inhibition of oxygen consumption, pyruvate utilization and citrate synthesis with concentrations about 10 to 20 times lower than were required with the diethylammonium and the sodium salts.

In the present investigation the in vitro effects of the sodium, diethylammonium and dimethylammonium salts of dithiocarbamic acid and bis(dimethyldithiocarbamyl) disulfide on alpha-ketoglutarate oxidation were studied prior to the conduction of assays on tissues of animals treated with the compounds. The in vitro studies were conducted by the addition of several concentrations of the compounds to the alpha-ketoglutarate oxidase system. When the per cent inhibition was plotted against the logarithm of the drug concentration, a straight line relationship resulted from which the concentration that would produce 50% inhibition of the enzyme activity was obtained. Oxygen consumption was measured as well as the amount of alpha-ketoglutarate utilized during the reaction period. The results of these measurements are summarized in Table 1. The results of these measurements demonstrated that the dimethylammonium, diethylammonium and sodium salts of dithiocarbamic acid as well as bis(dimethyldithiocarbamyl) disulfide are all effective inhibitors of alpha-ketoglutarate oxidation by rat liver. Measurements of the amount of alpha-ketoglutarate utilized and the amount of oxygen consumed showed essentially the same amounts of inhibition by the compounds and 50% inhibition of the reaction was obtained with concentrations which ranged from 1×10^{-4} M to 1×10^{-5} M for the various compounds.

The effect of the dimethylammonium salt of dithiocarbamic acid on the oxidation of pyruvate by homogenates of mouse and rat liver in vitro was also studied. Malonate was added to the test system. This results in the quantitative formation of acetoacetate from pyruvate by preventing the two-carbon fragments derived from pyruvate from entering the citric acid cycle. The results of these measurements demonstrated that the susceptibility of this reaction to inhibition by dimethylammonium dimethyldithiocarbamate is essentially the same as the susceptibility of the alpha-ketoglutarate system. The utilization of pyruvate in this system by rat and mouse liver homogenates was 50% inhibited by 7.4×10^{-5} M and 2.4×10^{-5} M dimethylammonium dimethyldithiocarbamate respectively. On the basis of oxygen consumption the reaction was

TABLE 1

INHIBITORY EFFECT OF DITHIOCARBAMIC ACID DERIVATIVES ON THE OXIDATION OF ALPHA-KETOGLUTARIC ACID BY RAT LIVER *IN VITRO*

Compound	Molar Concentration for 50% Inhibition	
	Oxygen Consumption	Alpha-ketoglutarate Utilization
Dimethylammonium dimethyldithiocarbamic acid	5.0×10^{-5}	2.6×10^{-5}
Diethylammonium diethyldithiocarbamic acid	7.6×10^{-5}	9.0×10^{-5}
Sodium diethyldithiocarbamic acid	1.2×10^{-4}	8.4×10^{-5}
Bis(dimethyldithiocarboxyl) disulfide	1.2×10^{-5}	2.0×10^{-5}

50% inhibited in mouse liver by 1.5×10^{-5} M indicating good agreement between the results obtained by measurements of oxygen consumption and pyruvate utilization.

The in vivo effect of dimethylammonium dimethyldithiocarbamate on the oxidation of alpha-ketoglutarate by the livers of rats, mice and guinea pigs. Following the conduction of the in vitro experiments described above, assays were performed on the livers of rats, mice and guinea pigs to ascertain the extent and duration of the inhibitory effect of dimethylammonium dimethyldithiocarbamate on the oxidation of alpha-ketoglutarate. A dose of 500 mgm./kgm. of the compounds was chosen for the initial studies because it approximates the maximum tolerated dose and is in the vicinity of the dosage level usually employed to obtain maximal radioprotective effects in rats. For this experiment groups each containing at least four rats were sacrificed at 0.5, 1, 2 and 3 hours after intraperitoneal injection of the compound for measurements of alpha-ketoglutarate oxidase activity. The amount of inhibition was estimated by measurement of oxygen consumption and chemical analysis of the amount of alpha-ketoglutarate utilized. The results obtained by the conduction of assays on the livers of rats at intervals within the first three hours after administration of 500 mgm./kgm. of dimethylammonium dimethyldithiocarbamate are shown in Table 2 in which the average and range of values for groups of four rats are presented.

The results of these experiments demonstrated that the dimethylammonium salt of dimethyldithiocarbamate is a potent inhibitor of the oxidation of alpha-ketoglutarate oxidase of rat liver in vivo. Nearly complete inhibition of the enzyme system was observed at 0.5 and 1 hour after intraperitoneal injection of the compound. Partial reversal of the inhibition was apparent in two hours and nearly complete reversal was noted in three hours.

To ascertain the effect of dimethylammonium dimethyldithiocarbamate on the alpha-ketoglutarate oxidase activity of the livers of mice 500 mgm./kgm. of the compound was given intraperitoneally and groups each containing four animals were sacrificed at intervals for five hours after drug treatment. The results of these measurements are summarized in Table 3 where it may be seen that marked inhibition of the enzyme activity was observed at 0.5 hours after injection of the compound. At one hour the enzyme system was approximately 50% inhibited. Nearly complete reversal of the inhibition occurred within two hours and normal activity was observed at three and five hours after drug treatment.

Further information on the response of various species to a given dose of dimethylammonium dimethyldithiocarbamate was obtained by the conduction of alpha-ketoglutarate assays on the livers of guinea pigs after intraperitoneal injection of 500 mgm./kgm. of the compound. Groups of four animals were sacrificed at 0.5, 1 and 2 hours after treatment with the compound. The results of these measurements are shown in Table 4. The greatest inhibitory effect was observed at 0.5 hours after administration of the compound as was the case with mice and rats. However, the maximum amount of inhibition of the enzyme activity in this species was less than that observed with the same dose in rats and mice. Rapid reversibility of the inhibition occurred as evidenced by activity approaching normal in two hours after injection of the compound. The results of these measurements demonstrated that dimethylammonium dimethyldithiocarbamate causes marked inhibition of alpha-ketoglutarate oxidase at a sublethal dosage

TABLE 2

EXTENT AND DURATION OF THE INHIBITORY EFFECT OF 500 MGM./KGM. OF DIMETHYLAMMONIUM DIMETHYLDITHIOCARBAMATE ON THE OXIDATION OF ALPHA-KETOGLUTARATE BY THE LIVERS OF RATS

Hours After Drug Injection	Oxygen Consumption		Alpha-ketoglutarate Utilization	
	Average Activity[a]	% Inhibition	Average Activity[a]	% Inhibition
Control	4.3 (3.8-4.9)	..	10.0 (9.2-10.9)	..
0.5	0.7 (0.5-0.7)	85	1.3 (1.2-1.4)	87
1.0	0.8 (0.2-2.0)	81	1.6 (1.5-1.6)	84
2.0	2.2 (1.4-4.5)	49	3.1 (2.3-3.8)	69
3.0	4.1 (3.8-5.2)	5	7.8 (5.7-9.3)	22

[a] Activity expressed as µM of oxygen consumed or alpha-ketoglutarate oxidized per 50 mgm. of tissue per 40 minutes.

TABLE 3

EXTENT AND DURATION OF THE INHIBITORY EFFECT OF 500 MGM./KGM. OF DIMETHYLAMMONIUM DIMETHYLDITHIOCARBAMATE ON THE OXIDATION OF ALPHA-KETOGLUTARATE BY THE LIVERS OF MICE

Hours After Drug Injection	Oxygen Consumption		Alpha-ketoglutarate Utilization	
	Average Activity[a]	% Inhibition	Average Activity[a]	% Inhibition
Control	6.4 (5.4-6.9)	..	13.0 (10.9-13.9)	..
0.5	1.1 (0.5-1.4)	83	2.8 (1.0-3.4)	78
1.0	3.3 (1.6-4.5)	48	6.0 (3.3-9.2)	54
2.0	5.5 (5.2-6.3)	14	11.0 (10.3-12.7)	15
3.0	6.2 (5.6-6.8)	3	11.6 (11.0-12.3)	11
5.0	6.6 (5.4-7.7)	0	12.2 (10.1-13.5)	6

[a] Activity expressed as μl of oxygen consumed or alpha-ketoglutarate oxidized per 50 mgm. of tissue per 40 minutes.

TABLE 4

EFFECT AND DURATION OF THE INHIBITION [illegible] OF 500 MG./KGM. OF METHYLAMPHETAMINE HYDROCHLORIDE ON THE OXIDATION OF ALPHA-KETOGLUTARATE BY THE LIVER OF WHITE RATS

Hours After Drug Injection	Oxygen Consumption		Alpha-ketoglutarate Utilization	
	Average Activity[a]	% Inhibition	Average Activity[a]	% Inhibition
Control	1.7 (1.5-1.9)	--	4.6 (4.4-5.1)	--
0.5	0.9 (0.6-1.1)	48	2.0 (1.7-2.3)	58
1.0	1.1 (0.8-1.3)	35	3.2 (2.8-3.4)	31
[illegible]	[illegible] (0.3-1.7)	[illegible]	[illegible] (3.8-5.0)	10

[a] Activity expressed as mg. of oxygen consumed or alpha-keto- [illegible] per 50 mg. of tissue per 60 minutes.

...el. Rats and mice were similar in their susceptibility and guinea pigs were somewhat more resistant.

Effect of bis(dimethyldithiocarbamyl) disulfide on the oxidation of alpha-ketoglutarate by rat liver. In vitro experiments (Table 1) indicated that bis(dimethyldithiocarbamyl) disulfide has the ability to inhibit the oxidation of alpha-ketoglutarate by rat liver homogenates and thus resembled salts of dimethyldithiocarbamic acid in inhibitory potency. Experiments were, therefore, undertaken to ascertain the effect of the maximum tolerated dose (150 mgm./kgm) of this compound on alpha-ketoglutarate oxidation in vivo. For this experiments groups each containing four rats were sacrificed at intervals ranging from 0.5 to 48 hours after injection of the compound for alpha-ketoglutarate oxidase assays on the liver. The results of these measurements are summarized in Table 5. Some inhibition of the enzyme activity occurred after administration of this compound with the maximum effect being observed at twelve hours. The results of these assays demonstrated that a maximum tolerated dose of bis(dimethyldithiocarbamyl) disulfide is less effective than dimethylammonium dimethyldithiocarbamate as an inhibitor of the oxidation of alpha-ketoglutarate and the time of onset and duration was much slower than was the case with the salts of dithiocarbamic acid.

Influence of dimethylammonium dimethyldithiocarbamate on the synthesis of acetoacetate from pyruvate. Since the enzyme system which catalyzes the decarboxylation of pyruvate is similar to the alpha-ketoglutarate oxidase system, it was anticipated that the dimethylammonium salt of dimethyldithiocarbamic acid would inhibit pyruvate oxidation. This possibility was demonstrated to be the case by in vitro experiments on mouse and rat liver homogenates. These tests were conducted with the addition of malonate to the test system which results in quantitative conversion to acetoacetate of the active acetate formed from pyruvate. Following the in vitro experiments assays were performed on the livers of rats and mice given 500 mgm./kgm. of dimethylammonium dimethyldithiocarbamate. Groups each containing four animals were sacrificed at intervals within the first seven hours after injection of the compound. Both oxygen consumption and pyruvate utilization were measured. The results of these measurements are summarized in Table 6. These assays demonstrated that 500 mgm./kgm. of dimethylammonium dimethyldithiocarbamate exerts a marked inhibitory effect on the oxidation of pyruvate. The maximal amount of inhibition occurred in one-half hour after injection of the compound in both rats and mice. In both species reversal of the inhibition began within two hours after injection of the compound. The inhibitory effect on pyruvate oxidation was essentially the same in mice and rats with respect to the amount of inhibition, the time of onset and the rate of reversal.

Discussion

The present study was a continuation of experiments which were recently undertaken in this laboratory (1,2) in an effort to obtain information on the mechanism responsible for the radioprotective activity and toxicity of dithiocarbamic acid derivatives and bis(dimethyldithiocarbamyl) disulfide. Our previous experiments indicated that the salts of dithiocarbamic acid and bis(dimethyldithiocarbamyl) disulfide inhibit one or more reactions of the tricarboxylic acid cycle in vitro. This inhibitory effect was detected by measuring the

TABLE 5

EFFECTS OF 150 MG./KGM. OF BIS(DIETHYLDITHIOCARBAMYL) DISULFIDE ON THE OXIDATION OF ALPHA-KETOGLUTARATE BY RAT LIVER

Hours After Drug Injection	Oxygen Consumption		Alpha-ketoglutarate to Succination	
	Average Activity[a]	% Inhibition	Average Activity[a]	% Inhibition
Control	4.3 (3.8-4.9)	-	10.0 (9.2-10.9)	-
0.5	3.1 (2.5-3.5)	28	6.8 (6.3-7.5)	32
1.0	3.7 (3.4-3.8)	14	8.3 (8.1-8.6)	17
3.0	2.6 (2.4-2.8)	40	4.7 (3.8-5.1)	53
5.0	3.0 (2.7-3.1)	30	5.8 (5.7-7.6)	42
12.0	2.3 (1.3-2.9)	47	4.9 (3.7-7.1)	51
24.0	4.2 (3.8-4.7)	2	9.3 (8.6-10.2)	7
48.0	3.8 (3.5-4.1)	12	8.6 (7.5-9.2)	14

[a] Activity expressed as µl of oxygen consumed or alpha-keto-glutarate oxidized per 50 mgm. of tissue per 30 minutes.

TABLE 6

THE INHIBITORY EFFECT OF 500 MGM./KGM. OF DIMETHYLAMMONIUM DIMETHYLDITHIOCARBAMATE ON THE OXIDATION OF PYRUVATE BY MOUSE AND RAT LIVER

Hours After Drug Injection	Oxygen Consumption		Pyruvate Utilization	
	Average Activity[A]	% Inhibition	Average Activity[A]	% Inhibition
Rats				
Control	1.7 (0.9-2.1)	..	4.0 (2.4-5.2)	..
0.5	0.2 (0-0.4)	88	0.5 (0.3-0.7)	88
1.0	0.3 (0-0.8)	78	1.2 (0.9-1.6)	70
2.0	0.7 (0.2-1.2)	59	2.0 (1.6-2.5)	50
3.0	0.8 (0.4-1.4)	53	2.0 (1.5-3.0)	50
5.0	1.0 (0.8-1.4)	41	2.6 (2.3-3.2)	35
Mice				
Control	2.1 (1.3-2.8)	..	4.9 (3.7-7.1)	..
0.5	0.3 (0-0.9)	86	1.2 (0.5-1.9)	76
1.0	0.3 (0-0.5)	86	1.1 (0.4-1.7)	78
2.0	0.2 (0-0.3)	90	1.2 (0.6-1.7)	76
5.0	0.9 (0.3-1.1)	57	2.5 (1.7-3.2)	49
7.0	1.5 (0.9-2.0)	29	3.7 (2.9-5.1)	24

[A] Activity expressed as μM of oxygen consumed or pyruvate utilized per 50 mgm. of liver per 10 minutes.

oxidation of pyruvate plus fumarate by homogenates of liver and kidney. When this combination of substrates is used the entire citric acid cycle is operative. Following this observation measurements of the in vivo effects of the dimethylammonium salt of dimethyldithiocarbamate and bis(dimethyldithiocarbamyl) disulfide on the activity of citric acid cycle enzymes was studied. The results of these measurements indicated that doses of dimethylammonium dimethyldithiocarbamate ranging from 166 to 1,000 mgm./kgm. caused marked inhibition of pyruvate utilization and citrate synthesis by the liver and kidney of rats when the animals were sacrificed at thirty minutes after the intraperitoneal injection of the compound. Rapid reversal of the inhibitory effect was noted as evidenced by return of the activity to normal levels within five hours after injection of the compound.

In contrast to the in vitro effects of bis(dimethyldithiocarbamyl) disulfide, maximum tolerated doses of this compound had no effect on the oxidation of pyruvate plus fumarate in vivo. This finding was interesting because this compound does not exhibit radioprotective activity. The results obtained in our previous studies (1,2) indicated that examination of the actions of dithiocarbamic acid derivatives on the individual enzymes of the tricarboxylic acid cycle would be worthwhile. In view of the high susceptibility of alpha-keto acid oxidases to inhibition by AET, as was demonstrated previously in this laboratory (9), it was of interest to investigate the influence of dithiocarbamates on the oxidation of alpha-ketoglutaric acid and pyruvate. In vitro measurements showed that the sodium, dimethylammonium and diethylammonium salts of dithiocarbamic acid are effective inhibitors of the oxidation of alpha-ketoglutarate. Bis(dimethyldithiocarbamyl) disulfide was also an effective inhibitor of this reaction in vitro. Following these observations the sublethal dose of 500 mgm./kgm. of the dimethylammonium salt of dithiocarbamic acid was given to rats, guinea pigs and mice and the animals were sacrificed at various intervals for alpha-ketoglutarate oxidase assays on the liver. Marked inhibition of the enzyme activity occurred in the livers of all species. The effect was most pronounced at 0.5 hours after injection and reversal of the inhibition began within two hours. Bis(dimethyldithiocarbamyl) disulfide also caused inhibition of the reaction but the effect was less pronounced and the time of onset of maximal inhibition was delayed as compared with the salts of dithiocarbamic acid.

The oxidation of pyruvate by the liver of rats was also markedly inhibited by dimethylammonium dimethyldithiocarbamate in vitro. Measurements of the in vivo effects of 500 mgm./kgm. of the compound on pyruvate oxidation demonstrated marked inhibition of the reaction in the livers of mice and rats. The amount of inhibition and the duration of the effect closely resembled the effect on alpha-ketoglutaric acid oxidase. The results of these studies have shown that dithiocarbamic acid derivatives inhibit two sulfhydryl enzymes involved in carbohydrate metabolism. Further studies are in progress to ascertain the effects of these radioprotective agents on other enzyme systems.

Summary

1. Measurements of the in vitro effects of three salts of dithiocarbamic acid and bis(dimethyldithiocarbamyl) disulfide on the oxidation of alpha-ketoglutarate by rat liver showed that all of these compounds cause 50% inhibition of the reaction at molar concentrations between 1 x 10^{-4} M and 1 x 10^{-5} M.

2. The oxidation of pyruvate by rat and mouse liver was 50% inhibited by concentrations of [illegible] x 10^{-5} M and 2.4 x 10^{-5} M dimethylammonium dimethyldithiocarbamate.

3. The in vivo effects of dimethylammonium dimethyldithiocarbamate on the oxidation of alpha-ketoglutaric acid was studied by the intraperitoneal administration of 500 mgm./kgm. of the compound to rats, mice and guinea pigs. Assays conducted on the livers at various intervals showed that marked inhibition of the reaction occurred within thirty minutes after injection and reversal of the inhibitory effects began within two hours. Bis(dimethyldithiocarbamyl) disulfide (150 mgm./kgm.) also inhibited this reaction but the effect was less pronounced and more delayed than in the case of the salt of dithiocarbamic acid.

4. The intraperitoneal administration of 500 mgm./kgm. of dimethylammonium dimethyldithiocarbamate caused marked inhibition of the oxidation of pyruvate by rat and mouse liver which was similar to the amount of inhibition of alpha-ketoglutarate oxidase system.

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PHARMACOLOGICAL AND TOXICOLOGICAL COMPOUNDS AS PROTECTIVE OR THERAPEUTIC AGENTS AGAINST RADIATION INJURY IN EXPERIMENTAL ANIMALS

I. The Influence of Various Chemical Compounds on Radiation Lethality in Mice

Vivian Flsak, Stanley Brois and John Doull

This report concerns: The survival time and mortality of CF_1 male mice treated with various chemical compounds immediately prior to the administration of a lethal dose of whole body x-irradiation.

Immediate or ultimate application of the results: To find chemical compounds capable of reducing injury or preventing mortality in x-irradiated animals. This study constitutes one phase of a screening program in which various chemicals, drugs and toxic agents are being tested against radiation lethality in an effort to find methods of modifying radiation injury. The detection of compounds capable of reducing radiation injury and the elucidation of their structure-activity relationships should be of value in understanding the basic mechanisms of radiation damage in animals.

* * * * * * * * *

Forty-seven compounds have been tested for prophylactic effectiveness against radiation lethality in mice during the past three months as a part of the radiation screening program of this laboratory. Three of these compounds showed significant protective effects in that they enabled mice to survive the 30-day post irradiation period which would otherwise have resulted in a 100% mortality. In addition, a number of the tested compounds increased the ST_{50} (median survival time) of mice. The chemicals included a variety of groups of compounds, most of which were related to previously tested agents, modified in such a manner to enable us to obtain, if possible, a better understanding of the protective action from the standpoint of structure-activity relationships.

Materials and Methods. Adult male Carworth Farms Mice (CF_1) weighing between 20 and 25 grams were employed for these studies. The animals were housed in air-conditioned rooms (75° F. to 80° F.) and were provided with food (Rockland House Pellets) and water ad libitum. All of the animals were kept under observation for at least one week prior to their use during which time those mice which failed to gain weight normally or which appeared to be unhealthy were removed and sacrificed. Both the control and experimental mice were selected at random from a single shipment of animals in order that their age and weight would be comparable.

To evaluate the radioprotective activity of these compounds, they were injected intraperitoneally into groups of ten mice which were exposed to 800 r of whole body x-irradiation 10 to 15 minutes later. Each compound was tested

for radioprotective activity at two or more dosage levels, one of which was near the maximum tolerated dosage level for the particular compound. Distilled water was used as the vehicle whenever possible and the concentration of solute to solvent was adjusted in each case so that a maximum of 2 cc of the body weight was injected. When necessary either dilute hydrochloric acid or sodium hydroxide were used in order to adjust the injection solution to a pH of approximately 7.0. Compounds incoluble in water were dissolved in propylene glycol, a mixture of propylene glycol and water, or suspended in a 0.5% solution of carboxymethyl cellulose. The control mice were injected with comparable amounts of the particular vehicle used in each instance and they were then simultaneously irradiated with the treated animals. Observations on the mortality of both the control and treated groups were made daily for a period of thirty days after the radiation exposure or until all of the mice in the treated group were dead.

The x-ray exposure was given as a single whole body exposure of 250 KVP, 15 ma. x-ray by means of a G.E. Maximar Therapy unit. The dose rate was determined prior to each irradiation period by means of a Victoreen Ionization Chamber (100 r thimble) and was found to be between 40 and 42 r per minute. The added filtration consisted of 0.25 mm. of copper and 1.0 mm. of aluminum and the target-skin distance was 75 cm. The animals were irradiated individually in plastic tubes (50 cc. centrifuge tubes provided with numerous air holes) which were placed radially on a rotating turntable so that each animal received an equal dose of x-irradiation.

The compounds designated by the code letter B were synthesized in this laboratory. The USAF code letter designation and the source of the other compounds is listed in Table 1. No attempt was made to verify the chemical structure of any of these compounds and they were not subjected to further purification prior to their use in the screening studies.

TABLE 1

SOURCE AND USAF CODE NUMBER OF COMPOUNDS
INCLUDED IN REPORT NO. 35

USAF Designation	Source of Compound
CS	Dr. E. B. Hodge, Commercial Solvents Corp., Terra Haute, Ind.
DO	Dr. A. W. Hart, Dow Chemical Company, Midland, Michigan
K	Dr. Gilbert Thiessen, Koppers Company, Inc., Pittsburgh 19, Pa.

Experimental

Preliminary toxicity studies. Prior to testing each of the compounds for radioprotective activity, it was necessary to determine the maximum amount

of each of the compounds which could be administered to mice without causing mortality due to chemical toxicity. Preliminary toxicity studies were, therefore, conducted in which small groups of mice were injected intraperitoneally with increasing dosage levels of each compound and the resulting mortality recorded during a period of one week. The approximate LD_{50} (7-day) thus obtained was used as the basis for selecting the dosage levels employed for the radiation screening tests.

It can be seen in Table 2 that some of the compounds included in this series were tested for radioprotective activity at dosage levels less than the maximum tolerated dosage levels suggested by the toxicity studies. Where compounds cause a severe depression, this effect combined with the fact that the mice are confined in rather close fitting tubes during the radiation exposure, is apt to cause excessive mortality. Hence, in these instances it was necessary to use a lower dose of drug. Recently we have constructed a temperature controlled box into which the mice are placed during the radiation exposure. By maintaining a temperature of approximately 76° F. to 78° F. during the radiation exposure period the overheating previously encountered has been reduced and this permits the use of dosage levels nearer the maximum tolerated dose for each of the compounds.

Radiation screening studies. The x-ray dose used for these studies (800 r) consistently produces a 100% mortality within a period of about two weeks in untreated CF_1 male mice under our experimental conditions. Radiation deaths usually first appear on the fifth to sixth day after radiation exposure, and the median survival time (ST_{50}) for the control animals is 9 ± 2 days. A compound is considered to exhibit significant radioprotective activity if it increases the ST_{50} by more than five days or if it permits any of the treated animals to survive for thirty days after the lethal exposure. The results of the radiation screening studies are presented in Table 2. Included in this table are the name and structural formula of each of the compounds, the vehicle used for both the toxicity and the radioprotective studies, the results of the preliminary toxicity studies, the dosage levels employed for the radioprotective studies, the increase or decrease (in days) in the ST_{50} of the treated mice in comparison with that of the simultaneously irradiated control animals and the mortality of the treated animals at thirty days after the x-ray exposure.

Five new isothiouronium derivatives were included in the present study. Of these only one, α-phenyl-β,β-dimethyl aminoethylisothiouronium sulfate (B-124), showed any beneficial effects as a radioprotective agent. When administered at a dose of 5 mgm./kgm., 10% of the mice given this compound survived the 30-day post irradiation period. Increasing the dose to 10 mgm./kgm. eliminated the protective effect. It is of interest that this particular derivative of the well known AET basic structure was considerably more toxic than most of the other compounds previously synthesized. Also of interest was the fact that the 1-isopropyl-2-(2-mercaptoethylamino)-4,4-dimethyl imidazolinium sulfate derivative (B-127), which was one of the least toxic of this group, was of no value as a radioprotective agent. This compound was already rearranged to a guanidinoethanethiol (GET) form in contrast to the other salts which presumably rearrange to that form in the body when administered in either a neutral or slightly alkaline medium. Figure 1 depicts graphically the percentage survival curves of B-124 at the two dosage levels employed.

TABLE 2

TOXICITY AND RADIO-PROTECTIVE ACTIVITY OF VARIOUS CHEMICAL COMPOUNDS

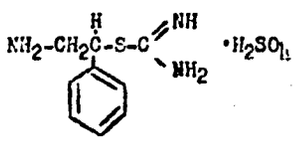
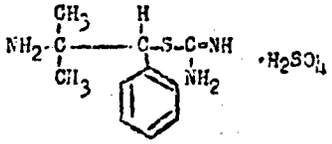
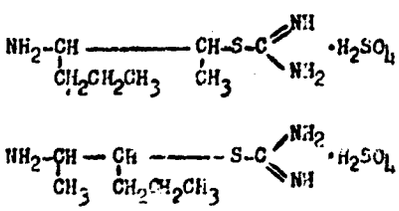
Compound (Name, Code Number, Vehicle, and Formula)	Toxicity	Radiation Studies		
	App. LD_{50} mgm./kgm.	Dose in mgm./kgm.	Change in ST_{50} in Days	Mortality 30 Days
α-Phenyl aminoethyliso-thiuronium sulfate B-123 (H_2O plus heat) 	25-50	25 10	- 1 - 3	10/10 10/10
α-Phenyl-β,β-dimethyl amino-ethylisothiuronium sulfate B-124 (H_2O plus heat) 	20-25	10 5	0 0	10/10 9/10
Mixture of: α-Methyl-β-propylaminoethyl-isothiouronium sulfate, and α-Propyl-β-methylaminoethyl-isothiouronium sulfate B-125 (H_2O)  	50-100	50 25	+ 1 0	10/10 10/10

TABLE 2--Continued

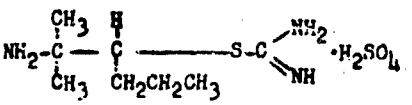
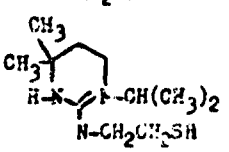
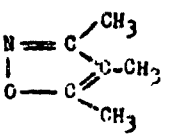
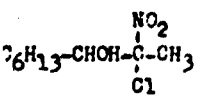
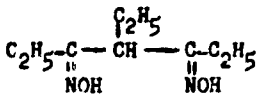
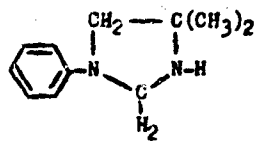
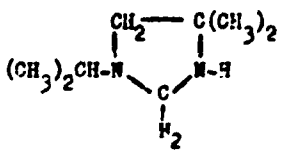
Compound (Name, Code Number, Vehicle, and Formula)	Toxicity	Radiation Studies		
	App. LD_{50} mgm./kgm.	Dose in mgm./kgm.	Change in ST_{50} in Days	Mortality 30 Days
α-Propyl-β,β-dimethyl amino-ethylisothiuronium sulfate B-126 (H_2O) 	10-25	10 5	- 1 - 1	10/10 10/10
1-Isopropyl-2-(2-mercaptoethyl-amino)-4,4-dimethyl imidazo-linium sulfate B-127 (H_2O) 	>1000	1000 500	- 2 - 2	10/10 10/10
3,4,5-Trimethylisoxazole CS-1340 (H_2O) 	>1000	500 300	0 - 4	10/10 10/10
2-Chloro-2-nitro-3-nonanol CS-1295 (PG) 	50-100	50 25	0 0	10/10 10/10

TABLE 2--Continued

Compound (Name, Code Number, Vehicle, and Formula)	Toxicity	Radiation Studies		
	App. LD_{50} mgm./kgm.	Dose in mgm./kgm.	Change in ST_{50} in Days	Mortality 30 Days
4-Ethyl-3,5-heptanedione dioxime CS-503 (PG) 	200-300	200 100	0 + 1	10/10 10/10
Propionohydroxamic acid CS-502 (H_2O) $C_2H_5CO-NHOH$	500	300 200	- 3 - 2	10/10 10/10
1-Phenyl-4,4-dimethylimidazolidine CS-1752 (PG) 	100-200	100 50	- 3 - 3	.....[a] 10/10
1-Isopropyl-4,4-dimethylimidazolidine CS-1751 (H_2O) 	300-500	300 200	+ 2 - 2	10/10 10/10

[a]Studies incomplete.

TABLE 2--Continued

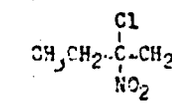
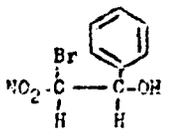
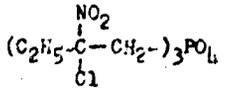
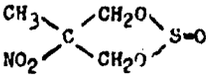
Compound (Name, Code Number, Vehicle and Formula)	Toxicity	Radiation Studies		
	App. LD_{50} mgm./kgm.	Dose in mgm./kgm.	Change in ST_{50} in Days	Mortality 30 Days
2-Chloro-2-nitro-1-butanol CS-257 (PG) 	200-300	200 100	0 2	10/10 10/10
2-Bromo-2-nitro-1-phenyl-1-ethanol CS-1301 (PG) 	25-50	25 10	- 9 + 1	10/10 10/10
Tris(2-chloro-2-nitro-1-butyl)phosphate CS-26 (PG) 	100-200	100 50	- 2 + 1	9/10 10/10
2-Nitro-2-methyl-trimethylene sulfite CS-399 (PG) 	300-500	300 200	+ 1 - 2	10/10 10/10

TABLE 2--Continued

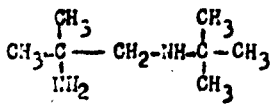
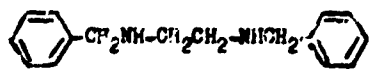
Compound (Name, Code Number, Vehicle, and Formula)	Toxicity	Radiation Studies		
	App. LD_{50} mgm./kgm.	Dose in mgm./kgm.	Change in ST_{50} in Days	Mortality 30 Days
Tris(propionoxymethyl)-nitromethane CS-1744 (PG) $(C_2H_5COO-CH_2-)_3C-NO_2$	300	200 100	0 + 2	10/10 10/10
N'-(t-Butyl-2-methyl-1,2-propanediamine CS-1745 (H_2O) 	200	100 50	- 4 0	10/10 10/10
3-Bromopropionitrile DO-51 (PG) $Br-CH_2-CH_2-CN$	50-100	50 25	+ 2 + 3	10/10 10/10
2,2'-(Methylimino) diethanol DO-52 (H_2O) $HOCH_2CH_2-N(CH_3)-CH_2CH_2OH$	500-1000	500 300	- 1 0	10/10 10/10
N,N'-Dibenzylethylene diamine DO-53 (PG) 	50-125	50 25	0 0	10/10 9/10

TABLE 2--Continued

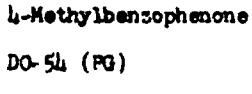
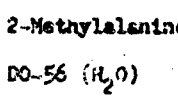
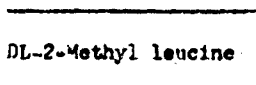
Compound (Name, Code Number, Vehicle, and Formula)	Toxicity	Radiation Studies		
	App. LD_{50} mgm./kgm.	Dose in mgm./kgm.	Change in ST_{50} in Days	Mortality 30 Days
4-Methylbenzophenone DO-54 (PG) 	250-500	250 100	- 2 0	10/10 10/10
Iminodiacetic acid DO-55 (H_2O plus heat) $HOOC-CH_2-NH-CH_2-COOH$	250-500	250 100	0 - 2	10/10 10/10
2-Methylalanine DO-56 (H_2O) 	> 1000	1000 500	- 1 - 2	10/10 10/10
DL-2-Methyl leucine DO-57 (H_2O) 	750-1000	750 500	+ 1 + 1	10/10 10/10

TABLE 2--Continued

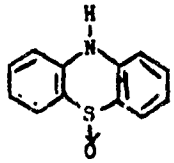
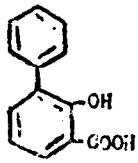
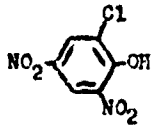
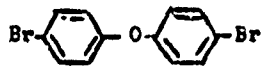
Compound (Name, Code Number, Vehicle, and Formula)	Toxicity	Radiation Studies		
	App. LD_{50} mgm./kgm.	Dose in mgm./kgm.	Change in ST_{50} in Days	Mortality 30 Days
Phenothiazine-5-oxide DO-58 (PO) 	50-100	750 500	+ 2 + 1	10/10 10/10
3-Phenylsalicylic acid DO-59 (PO) 	125-250	100 50	- 2 - 3	10/10 10/10
2-Chloro-4,6-dinitrophenol DO-60 (PO) 	125-250	25 10	- 3 + 2	10/10 10/10
Bis-(p-bromophenyl)ether DO-61 (PO) 	125-250	100 50	- 1 + 2	10/10 10/10

TABLE 2--Continued

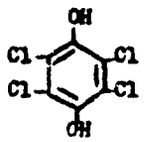
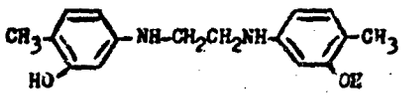
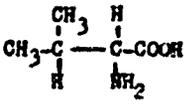
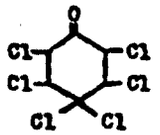
Compound (Name, Code Number, Vehicle, and Formula)	Toxicity	Radiation Studies		
	App. LD_{50} mgm./kgm.	Dose in mgm./kgm.	Change in SF_{50} in Days	Mortality 30 Days
Tetrachlorohydroquinone DO-62 (PG) 	25-50	10 5	- 2 0	10/10 10/10
N,N'-Ethylenediimino di(o-cresol) DO-63 (PG) 	100-200	100 50	+ 1 + 1	10/10 10/10
DL-Isovaline DO-64 (H_2O plus heat) 	>1000	1000 500	- 2 + 1	10/10 10/10
Hexachloro-2,5-cyclohexadien-1-one DO-65 (PG) 	50-100	10 5	+ 1 + 1	10/10 10/10

TABLE 2—Continued

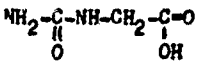
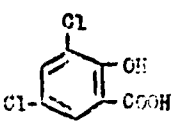
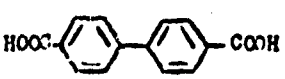
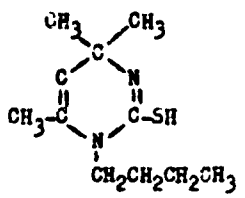
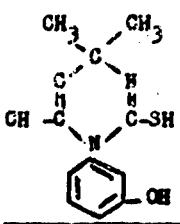
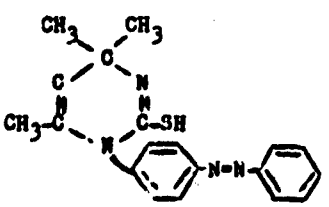
Compound (Name, Code Number, Vehicle, and Formula)	Toxicity	Radiation Studies		
	App. LD_{50} mgm./kgm.	Dose in mgm./kgm.	Change in ST_{50} in Days	Mortality 30 Days
Hydantoin DO-66 (H_2O plus heat) 	>1000	1000 500	+ 1 - 3	10/10 10/10
Hydantoic acid DO-67 (H_2O plus heat) 	>1000	500 300	- 5 0	10/10 10/10
3,5-Dichlorosalicylic acid DO-68 (PO) 	50-100	50 25	0 + 1	10/10 10/10
4,4'-Biphenyldicarboxylic acid DO-69 (H_2O and $NaHCO_3$) 	500-1000	500 300	- 2 + 2	10/10 10/10

TABLE 2--Continued

Compound (Name, Code Number, Vehicle, and Formula)	Toxicity	Radiation Studies		
	App. LD_{50} mgm./kgm.	Dose in mgm./kgm.	Change in ST_{50} in Days	Mortality 30 Days
1-(n-Butyl)-2-thio-4,4,6-trimethyldihydropyrimidine K-1339 (PG) 	100-200	100 50	+ 2 - 1	10/10 10/10
1-(m-Hydroxyphenyl)-2-thio-4,4,6-trimethyl dihydropyrimidine K-1345 (PG) 	100-200	100 50	+ 1 - 1	10/10 10/10
1-[p-(Phenylazo)phenyl]-2-thio-4,4,6-trimethyl dihydropyrimidine K-1354 (PG) 	200-300	200 100	- 2 0	.....[a] 10/10

[a]Studies not complete.

TABLE 2--Continued

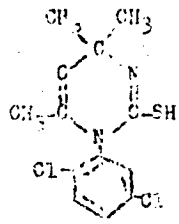
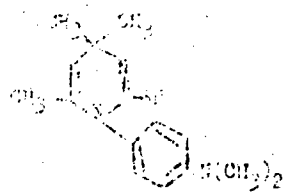
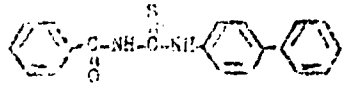
Compound (Name, Code Number, Vehicle and Formula)	Toxicity	Radiation Studies		
	App. LD_{50} mgm./kgm.	Dose in mgm./kgm	Change in SF_{50} in Days	Mortality 30 Days
1-(2,5-Dichlorophenyl)-2-thio-4,4,6-trimethyl dihydro-pyrimidine X-1350 (PG) 	100-200	100 50	- 2 0	10/10 10/10
1-(p-Dimethylaminophenyl)-2-thio-4,4,6-trimethyl dihydropyrimidine X-1352 (PG) 	200	100 50	- 2 - 1	10/10 10/10
2-Phenyl-1-(p-biphenyl)-2-thiourea X-1250 (PG) 	100-200	100 50	- 1 0	10/10 10/10

TABLE 2--Continued

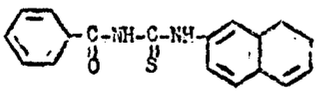
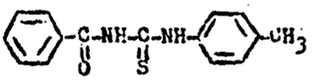
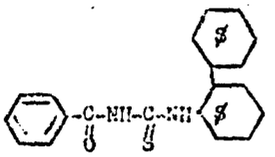
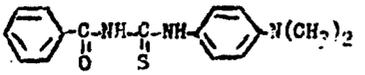
Compound (Name, Code Number, Vehicle, and Formula)	Toxicity	Radiation Studies		
	App. LD_{50} mgm./kgm.	Dose in mgm./kgm.	Change in ST_{50} in Days	Mortality 30 Days
3-Benzoyl-1-(β-naphthyl)-2-thiourea K-1475 (PG) 	200-300	200 100	- 1 0	10/10 10/10
3-Benzoyl-1-(p-tolyl)-2-thiourea K-1470 (PG) 	100-200	100 50	- 3 0	10/10 10/10
3-Benzoyl-1-(o-bicyclohexyl)-2-thiourea K-1468 (PG) 	100-200	100 50	- 1 0	10/10 10/10
3-Benzoyl-1-(p-dimethylaminophenyl)-2-thiourea K-1467 (PG) 	50-100	50 25	- 1 0	10/10 10/10

TABLE 2--Continued

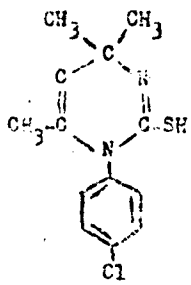
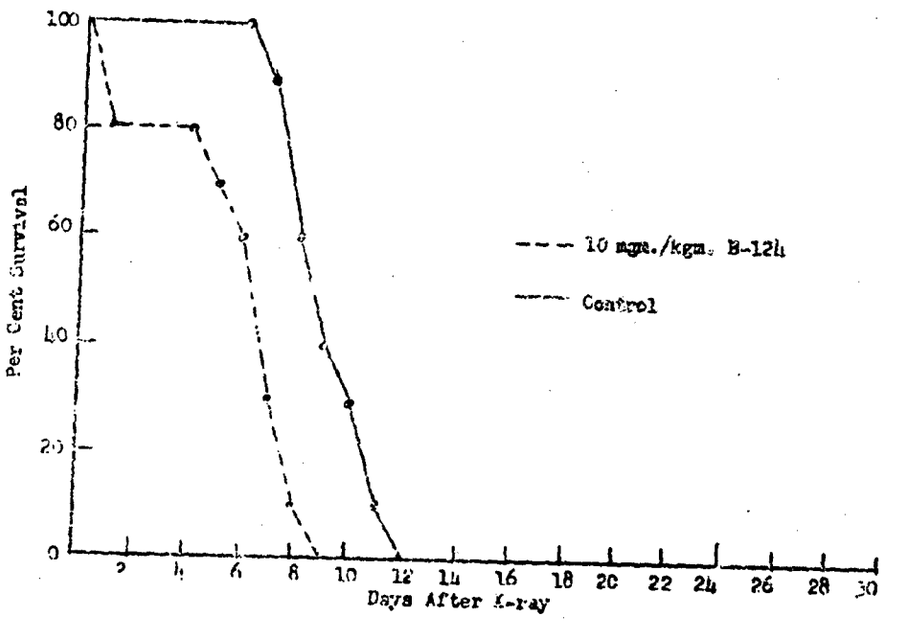
Compound (Name, Code Number, Vehicle, and Formula)	Toxicity	Radiation Studies		
	App. LD_{50} mgm./kgm.	Dose in mgm./kgm.	Change in ST_{50} in Days	Mortality 30 Days
1-(p-Chlorophenyl)-2-thio-4,4,6-trimethyl dihydro-pyrimidine K-1349 (PG) 	200	100 50	- 1 + 1	10/10 10/10

Figure 1

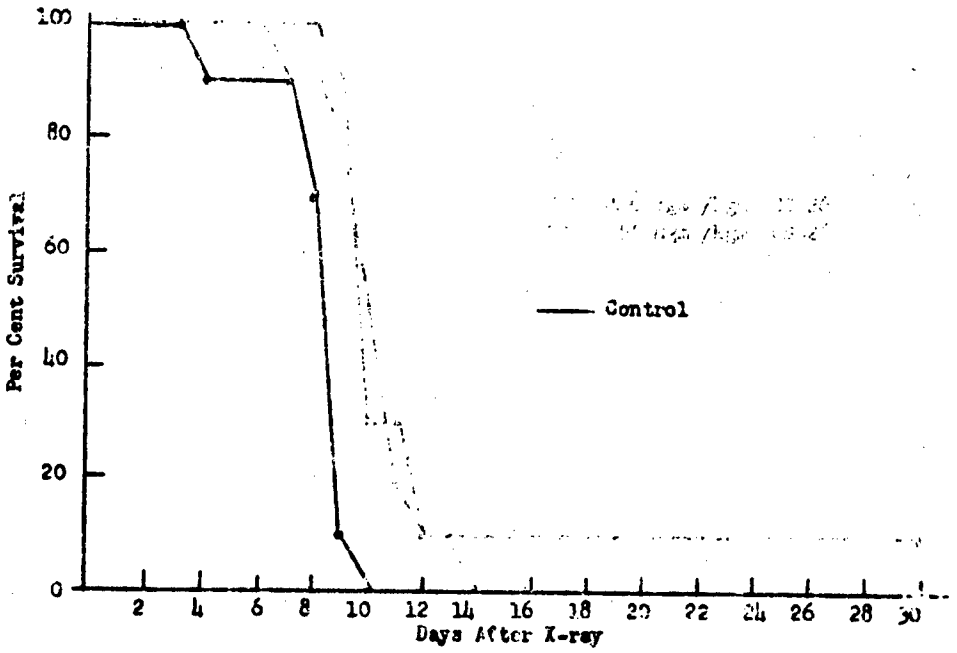
Effect of α-Phenyl-β,β-dimethyl aminoethyl isothiuronium sulfate (B-124) on Survival of Mice Irradiated with 800 r of Whole Body X-irradiation



A miscellaneous group of aliphatic and aromatic alcohols and amines, with or without nitro and chloro substituents, had been previously included in our screening program (1). Since some of these compounds exhibited slight to moderate radioprotective activity, a number of compounds of similar structure were included in this study. However, only tris(2-chloro-2-nitro-1-butyl) phosphate (CS-26) and N,N'-dibenzylethylene diamine (DO-53) showed any radioprotective effects. CS-26 permitted 10% of the mice to survive for thirty days after irradiation when administered at 1,000 mgs./kgs. and DO-53 did likewise at 25 mgs./kgs. With the former compound a reduced dose eliminated the protective effect while in the latter case it was the lowest dose of the two tested which protected. Figure 2 shows the aforementioned results graphically.

Figure 2

Effect of Tris(2-chloro-2-nitro-1-butyl)phosphate (CS-26) and N,N'-Dibenzylethylene diamine (DO-53) on Survival of Mice Irradiated with 800 r of Whole Body X-irradiation



Summary

Forty-seven chemical compounds have been tested in the present study for evidence of protective activity against radiation lethality in mice. Of these significant radioprotective effects were obtained with α-phenyl-β,β-dimethylaminoethylisothiuronium sulfate (B-124), tris(2-chloro-2-nitro-1-butyl) phosphate (CS-26) and with N,N'-dibenzylethylene diamine (DC-53). The radioprotective effect of the isothiuronium derivative is much less than that of the parent compound aminoethylisothiuronium (AET) but it is of interest since only a few of the AET derivatives tested in this program have exhibited protective effects. Neither of the two other compounds which exhibited protective activity contain sulfur. One of these compounds (CS-26) is of particular interest since it represents the first phosphate derivative which we have tested that exhibits radioprotective activity. It is planned to investigate additional compounds of this type.

Reference

1. Doull, J., Pizak, V., and Brois, S. J., USAF Radiation Laboratory Screening Program Status Report No. 1, February 1, 1959.

PHARMACOLOGICAL AND TOXICOLOGICAL COMPOUNDS AS PROTECTIVE OR THERAPEUTIC AGENTS AGAINST RADIATION INJURY IN EXPERIMENTAL ANIMALS

II. Further Studies on the Mechanism of the Radioprotective Action of Serotonin

B. J. Tricou and J. Doull

This report concerns: The survival time, mortality and weight loss of mice treated with serotonin prior to the administration of varying doses of whole body x-irradiation and the influence of the time of administration of the serotonin, environmental temperature during irradiation and administration of serotonin antagonists on the radioprotective activity of serotonin against radiation lethality in mice.

Immediate or ultimate application of the results: These studies were conducted to obtain information on the mechanism(s) by which serotonin protects mice against the injurious actions of whole body x-irradiation. A better understanding of the radioprotective effect of this compound would be of considerable value in the selection of related compounds for synthesis and testing as radioprotective agents. Such information should also provide increased insight into the nature of radiation injury itself.

* * * * * * * * *

In previous studies (1,2) we demonstrated that serotonin is a highly effective radioprotective agent in mice thus confirming a previous report of protective activity in this species (3). Dosage levels of 75 to 100 mgm./kgm. of the serotonin creatinine sulfate complex (ASP-1) permitted 70% to 80% of the pretreated animals to survive a lethal dose of whole body x-irradiation (800 r) for at least thirty days. In further studies (1) we were also able to demonstrate that the radioprotective effect of this compound can be substantially reduced by subjecting the mice to five atmospheres of oxygen pressure during the radiation exposure. Similar results have been reported in rats by Van den Brenk (5) who also obtained some reversal of the radioprotective effects of serotonin in rats by the use of serotonin antagonists. It was of interest to determine whether the radioprotective activity of serotonin in mice could be reversed by known serotonin antagonists. Four such antagonists have been obtained and the present study contains the results of measurements of the effects of these compounds on the radioprotective activity of serotonin in mice.

Hypothermia has been reported to exert radioprotective effects in animals (6) and since it is possible that the radioprotective activity of serotonin may be related to its hypothermic activity, we have investigated the effect of serotonin administration on the rectal temperature of mice exposed to increased and decreased environmental temperatures before and during radiation exposure.

In previous studies (2) it was observed that the ability of serotonin to prevent the radiation-induced increase in the adenosine triphosphatase activity of the spleens of x-rayed mice was more pronounced when low doses (100 to 300 rep) of radiation were employed. It appeared from these studies that the radioprotective activity of serotonin is related to the radiation dose administered. To further investigate this effect studies were carried out in which the radioprotective activity of this compound was measured throughout a range of x-ray exposures. Probit mortality data from these studies has been analyzed statistically and is presented in this report.

We have previously demonstrated (2) that the optimal time to administer serotonin to obtain maximal radioprotective activity was immediately prior to the radiation exposure. Since it required about twenty minutes to administer the radiation dose in these studies, it is difficult to evaluate the influence of the duration of the pharmacologic effect of serotonin in relationship to the time of administration. It was felt that more precise information could be obtained by administering the radiation dose in as short a time as possible and in the present study we have investigated the influence of the time of administration of the serotonin when the radiation exposure is given in a very short time (approximately one minte).

Materials and Methods. Adult male and female Carworth Farms mice (CF_1) weighing between 20 and 25 grams were employed for these studies. The animals were housed in an air-conditioned room (75° F. to 80° F.) and were provided with food (Rockland Mouse Pellets) and water ad libitum. Aqueous solutions of the serotonin creatinine sulfate (ASP-1) and of the other drugs were administered intraperitoneally 5 to 10 minutes prior to the radiation exposure except as otherwise specified.

All of the x-ray exposures were given as single whole body irradiations by means of a G.E. X-ray Unit (250 KVP, 15 ma.) at a target distance of 75 cm. and a dose rate of 39 to 41 r per minute. The dose rate was determined prior to each irradiation experiment by means of a 100 r Victoreen Ionization Thimble in air. The added filtration consisted of 0.25 mm. of copper plus 1.0 mm. of aluminum. In the high dose rate studies the target skin distance was about 20 cm. and the dose rate was 570 r per minute. There was no added filtration present in these studies. Daily observation of the mortality and weight changes were made in all of the control and experimental groups.

Rectal temperatures were measured by means of a thermistor (Veco Type 32A11) connected to an appropriate bridge circuit. The sensitivity of this apparatus is about 0.2° F. and the response time is less than two seconds. The environmental temperature of the animals was maintained at 26° C. during the radiation exposure by means of an insulated temperature controlled box. In those experiments in which higher and lower environmental temperatures were tested, the temperature within this box was controlled thermostatically during the radiation exposure to either 4° C. or 38° C. and the mice were pre-chilled or pre-warmed for twenty minutes prior to the irradiation exposure.

The sample of serotonin creatinine sulfate (ASP-1) and the antagonist (SA-97)* used for these studies was supplied by Dr. K. Schoch, Abbott Laboratories, North Chicago, Illinois. The d-lysergic acid diethylamide (LSD-25)

*N-methyl-N'-(4-chlorobenzhydryl)-1,4-diazacycloheptane.

and d-bromlysergic acid diethylamide (BOL-148) were provided by Dr. R. Bircher, Sandoz Inc., Hanover, New Jersey and the sample of methergine was obtained commercially.

Experimental

Effect of time of administration of serotonin in relation to lethal doses of whole body irradiation on the survival time of mice. In previous studies (2) it was found that the maximum effectiveness of serotonin against lethality in mice exposed to 800 r of whole body x-irradiation occurred when the drug was injected within ten minutes prior to the radiation exposure. Since the radiation exposure occurred over a 20-minute period, it was desirable to determine the optimal period of activity of the drug more precisely by reducing the time of the irradiation exposure. A dose of 646 r was found to be adequate to just kill all controls and allow the manifestation of varying degrees of the protective action of serotonin when the radiation was given at a dose rate of 570 r/minute. This dose was administered in a period of 68 seconds. Male CF_1 mice were injected intraperitoneally with an aqueous solution of serotonin creatinine sulfate at a dose level of 90 mgm./kgm. at 10, 20, 45 and 60 minutes prior to the irradiation. The rectal temperature of these mice was measured just prior to the irradiation and at intervals after the radiation exposure. At least sixteen animals were used in each group. The results can be seen in Figure 1. The maximal 30-day survival was obtained when the drug was administered at 10 to 20 minutes prior to irradiation, although the maximum decrease in body temperature occurred at 60 minutes after the serotonin injection. Another group of animals was given serotonin immediately after the radiation exposure was completed and these mice showed no difference in survival time or mortality as compared with animals receiving no drug.

Influence of environmental temperature on radioprotective effect of serotonin. In order to investigate the effect of the hypothermic properties of serotonin on the mechanism of its radioprotective activity, ten groups of sixteen CF_1 male mice were irradiated at three temperatures. Control mice and mice injected intraperitoneally with 90 mgm./kgm. of serotonin were irradiated at room temperature (25° C.), at 4° C., at 4° C. after a 20-minute period of chilling, at 38° C. and at 38° C. after a 20-minute period of warming. Rectal temperatures were measured immediately prior to and immediately after the exposure to 800 r of whole body x-irradiation at a dose rate of 40 r/minute. The results can be seen in Figures 2 and 3 and in Table 1. It can be seen that the temperature during and prior to irradiation had no effect on the mortality or weight loss of control mice and of those mice treated with serotonin. The mice kept at and irradiated at room temperature showed 75% survival at the end of thirty days, whereas those animals which were chilled and attained a rectal temperature of 5° to 10° C. lower than the controls exhibited only 50% to 53% survival at thirty days. The mice which were warmed and irradiated in a warm atmosphere showed a high degree of initial drug toxicity. Of the survivors from the initial effects of irradiation, survival at thirty days was intermediate between those animals irradiated at room temperature and those which were chilled. It may, therefore, be concluded that in these ranges the radioprotective effect of serotonin is not due to the lowering of body temperature in mice.

Figure 1

Rectal Temperature (Pre-irradiation) and 30-Day Survival (Post-irradiation) of Mice Given 90 mgm./kgm. of Serotonin at Various Intervals Before a Lethal Exposure to Whole Body X-irradiation

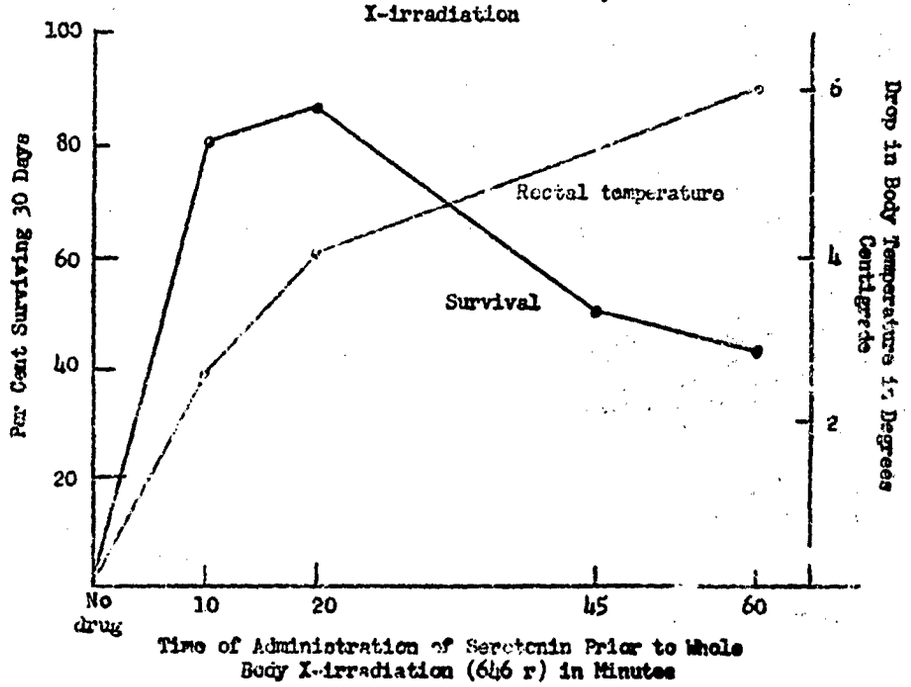


Figure 2. Influence of Environmental Temperature During Irradiation Upon Survival and Weight Loss of CF_1 Male Mice Exposed to 800 r of Whole Body X-irradiation

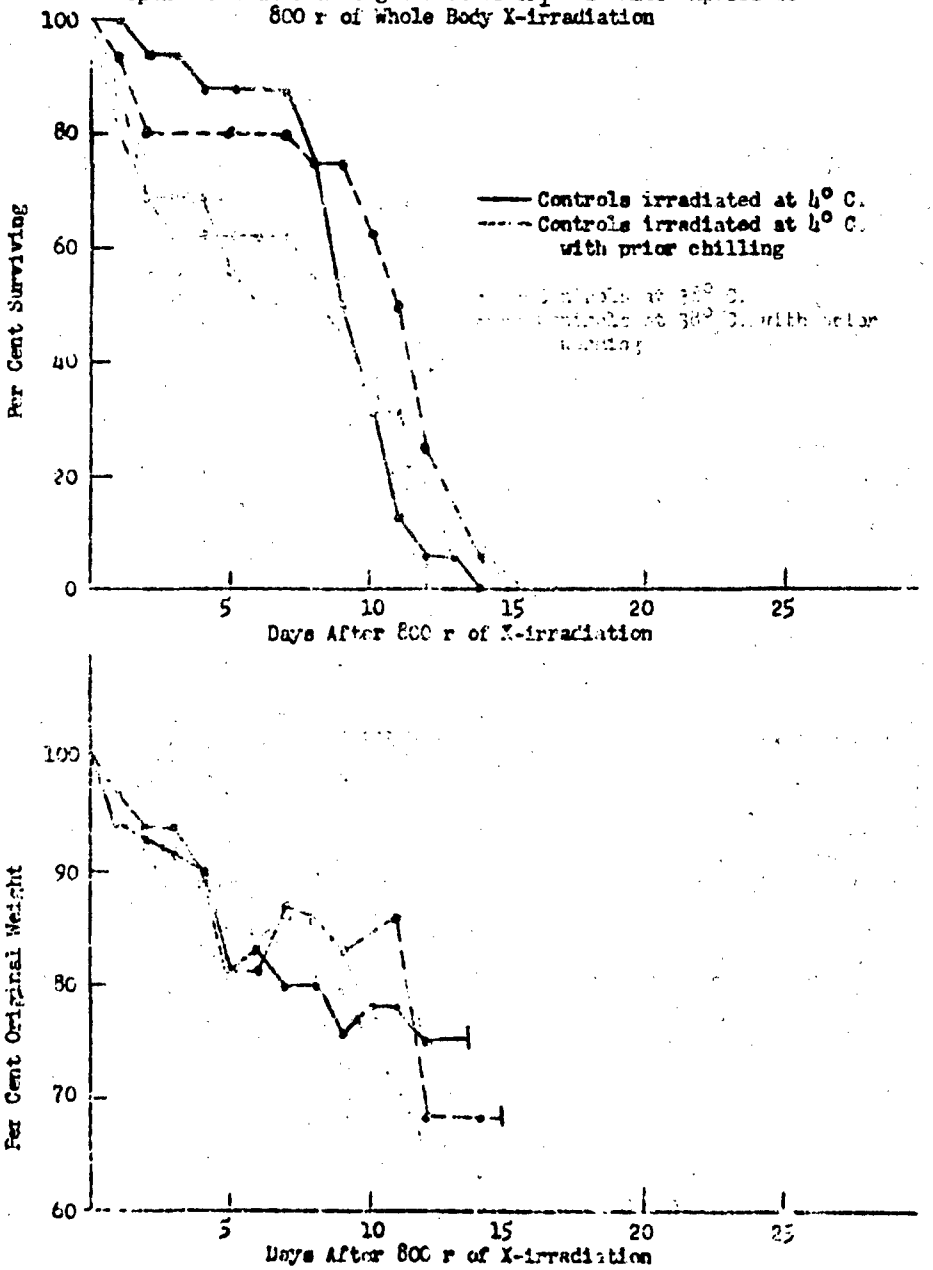


Figure 3. Influence of Environmental Temperature During Irradiation on the Radioprotective Effects of Serotonin Against Lethality and Weight Loss in CF_1 Males After 800 r of X-ray

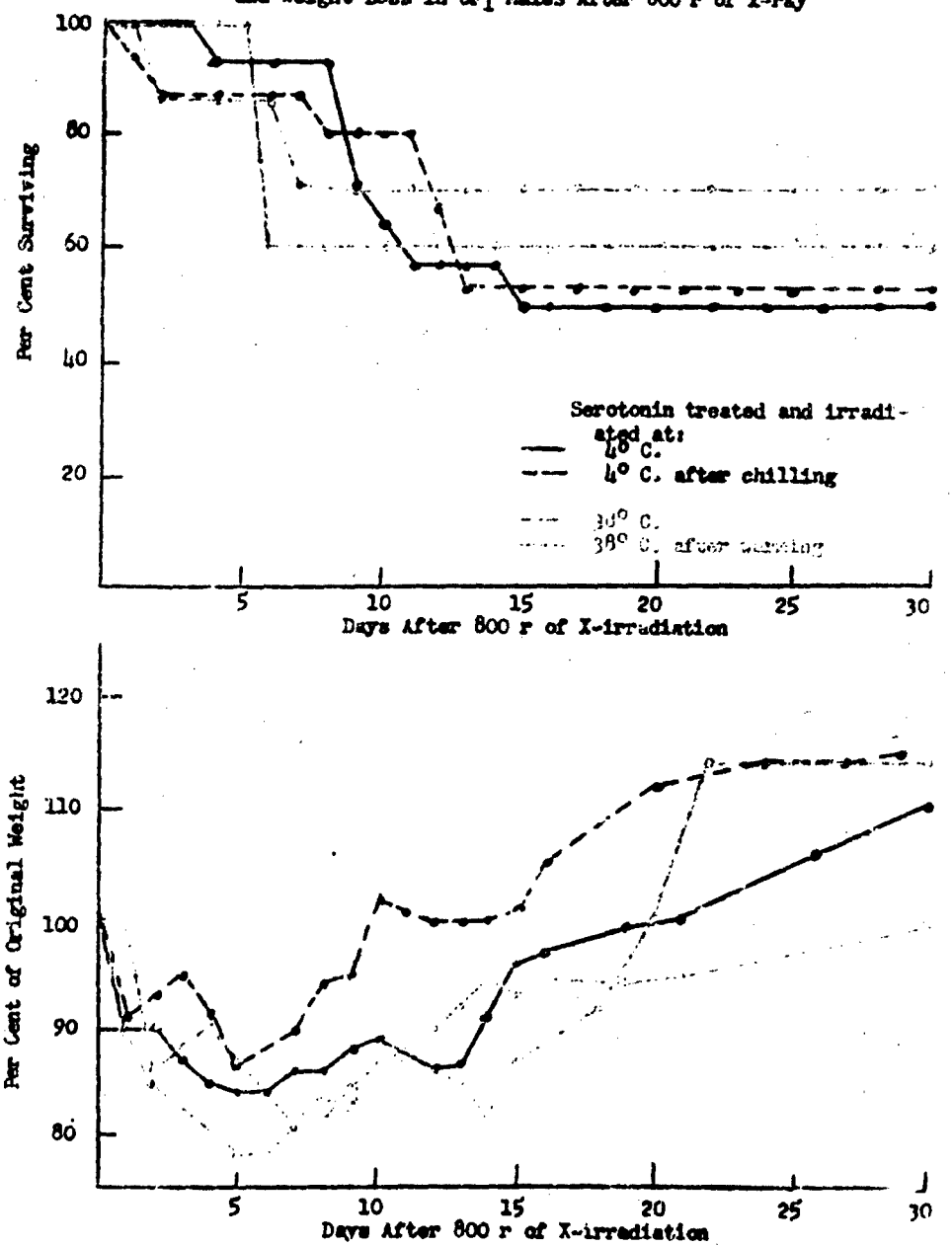


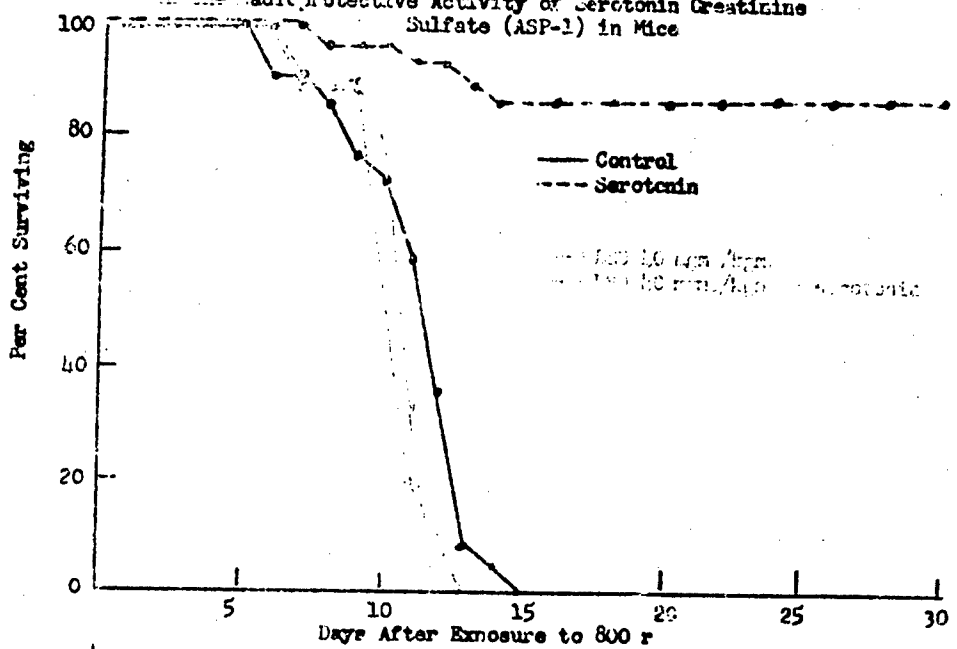
TABLE 1

RECTAL TEMPERATURE OF MICE EXPOSED TO VARYING ENVIRONMENTAL TEMPERATURES BEFORE AND DURING A LETHAL X-RAY EXPOSURE

	Environmental Temperature During X-ray	Environmental Temperature Prior to X-ray	% Surviving at 30 Days	ST_{50} (Days)	Mean Rectal Temperature
Controls	4° C.	25° C.	0	9.0	36.7° C.
	4° C.	10° C.	0	10.3	36.8° C.
	25° C.	25° C.	0	10.0	38.1° C.
	38° C.	25° C.	0	9.0	39.1° C.
	38° C.	38° C.	0	9.0	39.3° C.
Serotonin-treated	4° C.	25° C.	50	....	32.4° C.
	4° C.	10° C.	53	....	27.4° C.
	25° C.	25° C.	75	....	36.2° C.
	38° C.	25° C.	60	....	39.0° C.
	38° C.	38° C.	71	....	38.6° C.

Effect of administration of serotonin antagonists on the radioprotective effects of serotonin in mice. It has been reported that LSD, BOL-148 and BAS phenol administered prior to serotonin block the radioprotective effect of the latter compound in rats (4). In view of the conclusion implicit in these results that the radioprotective effect is related to the pharmacologic effect of serotonin, it was desirable to study these compounds and others in relation to the radioprotective effect of serotonin in mice. LSD, BOL-148, Methergine (methyl ergonovine), and an experimental serotonin antagonist SA-97 were selected for study. Male CF_1 mice in groups of ten animals were injected with the antagonists at two dose levels; after thirty minutes half the groups received serotonin and all of the groups were then exposed to an x-ray dose of 800 r at a dose rate of 40 r/minute. The results may be seen in Figures 5 through 7. In no case did the antagonists show protective effects alone, and in the case of BOL-148, at least, there was some hastening of mortality. LSD-25 and BOL-148 completely obliterated the radioprotective effect of serotonin at the higher dose level and significantly depressed it at dosages of 5 mgm./kgm. of antagonist. SA-97 diminished the radioprotective effect of serotonin

Figure 4. Effect of D-Lysergic Acid Diethylamide Tartrate (LSD-25) on the Radioprotective Activity of Serotonin Creatinine Sulfate (ASP-1) in Mice



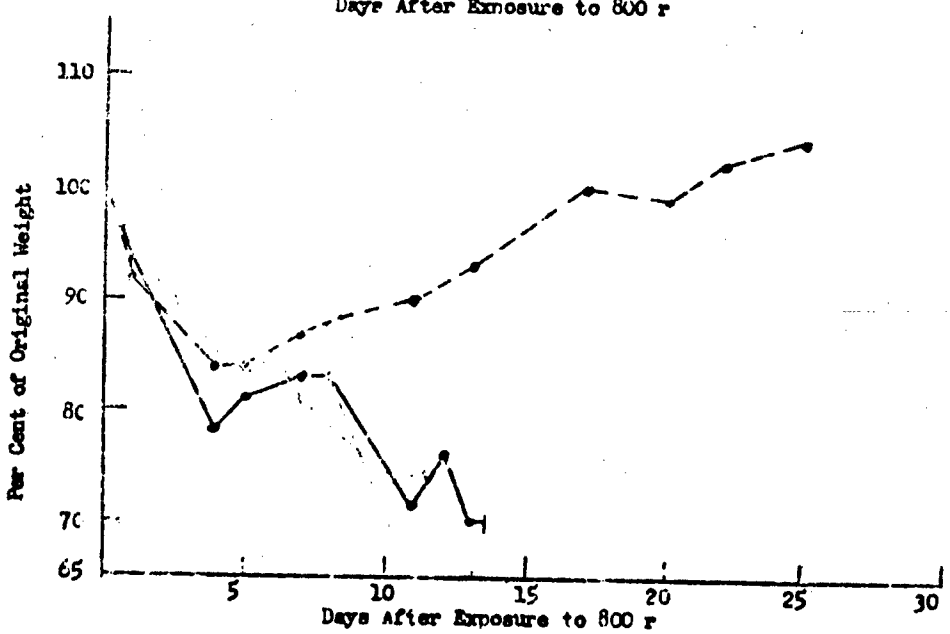


Figure 5. Effect of d-Brom Lysergic Acid Diethylamide (BOL-148) on the Radioprotective Activity of Serotonin Creatinine Sulfate (ASP-1) in Mice

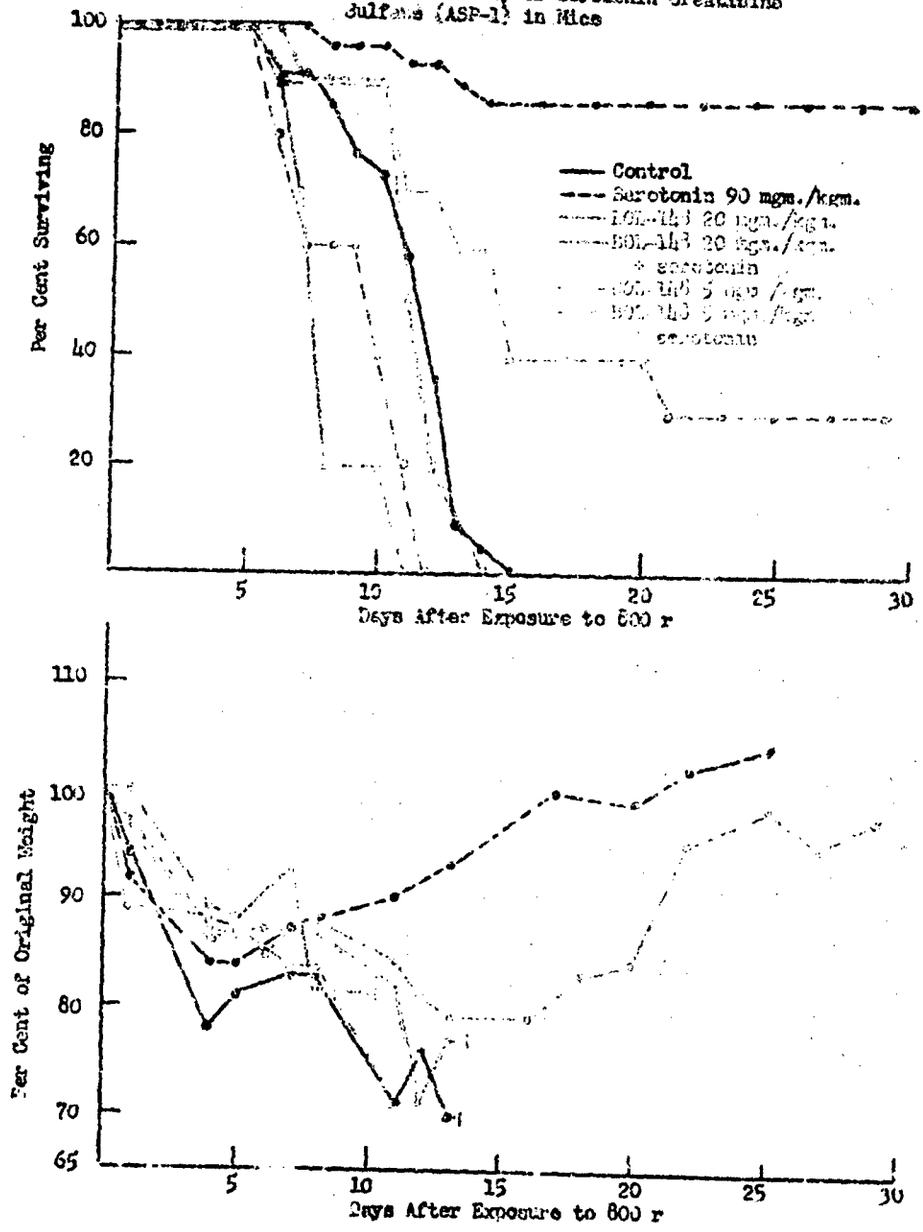


Figure 6. Effect of the Serotonin Antagonist SA-97 on the Radioprotective Activity of Serotonin Creatinine Sulfate (ASP-1) in Mice

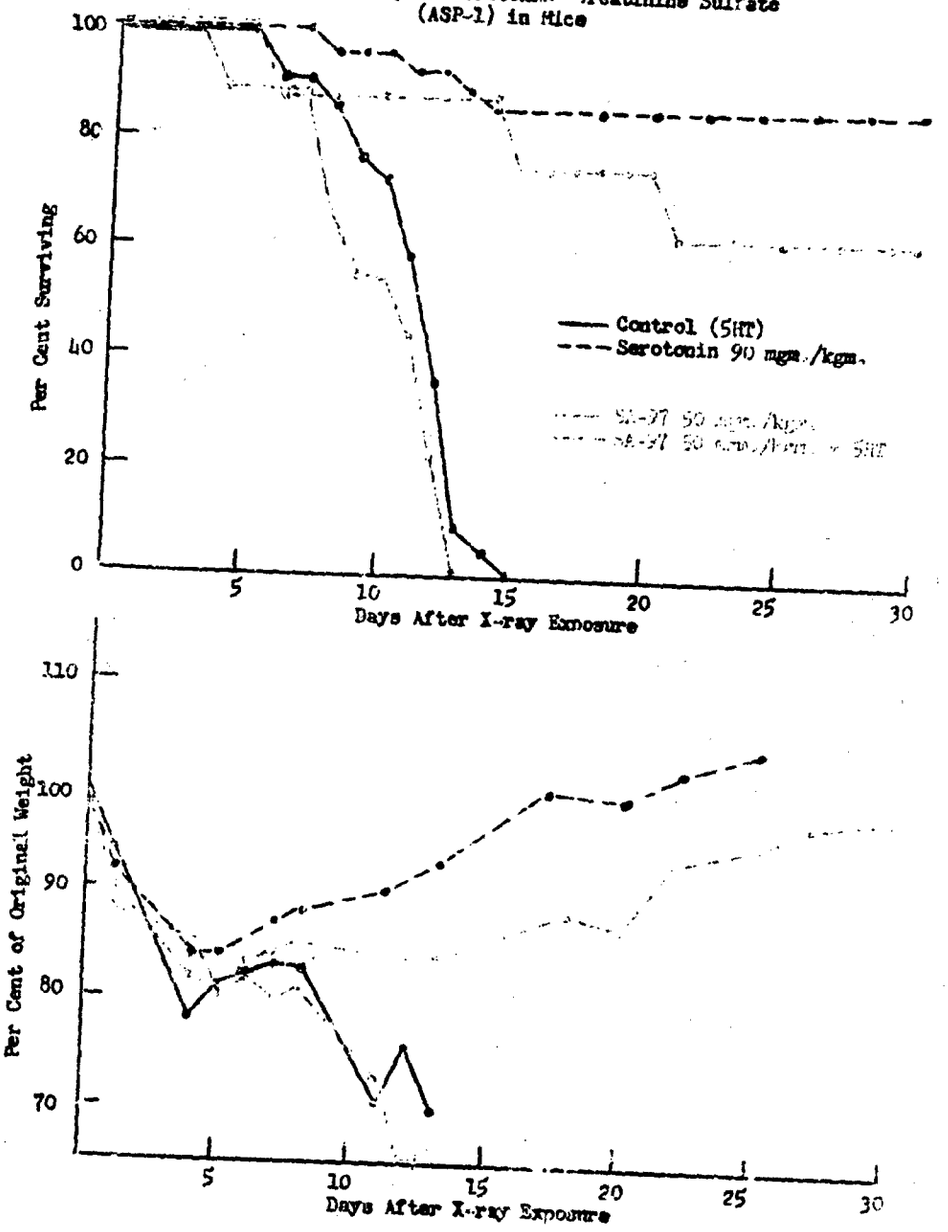
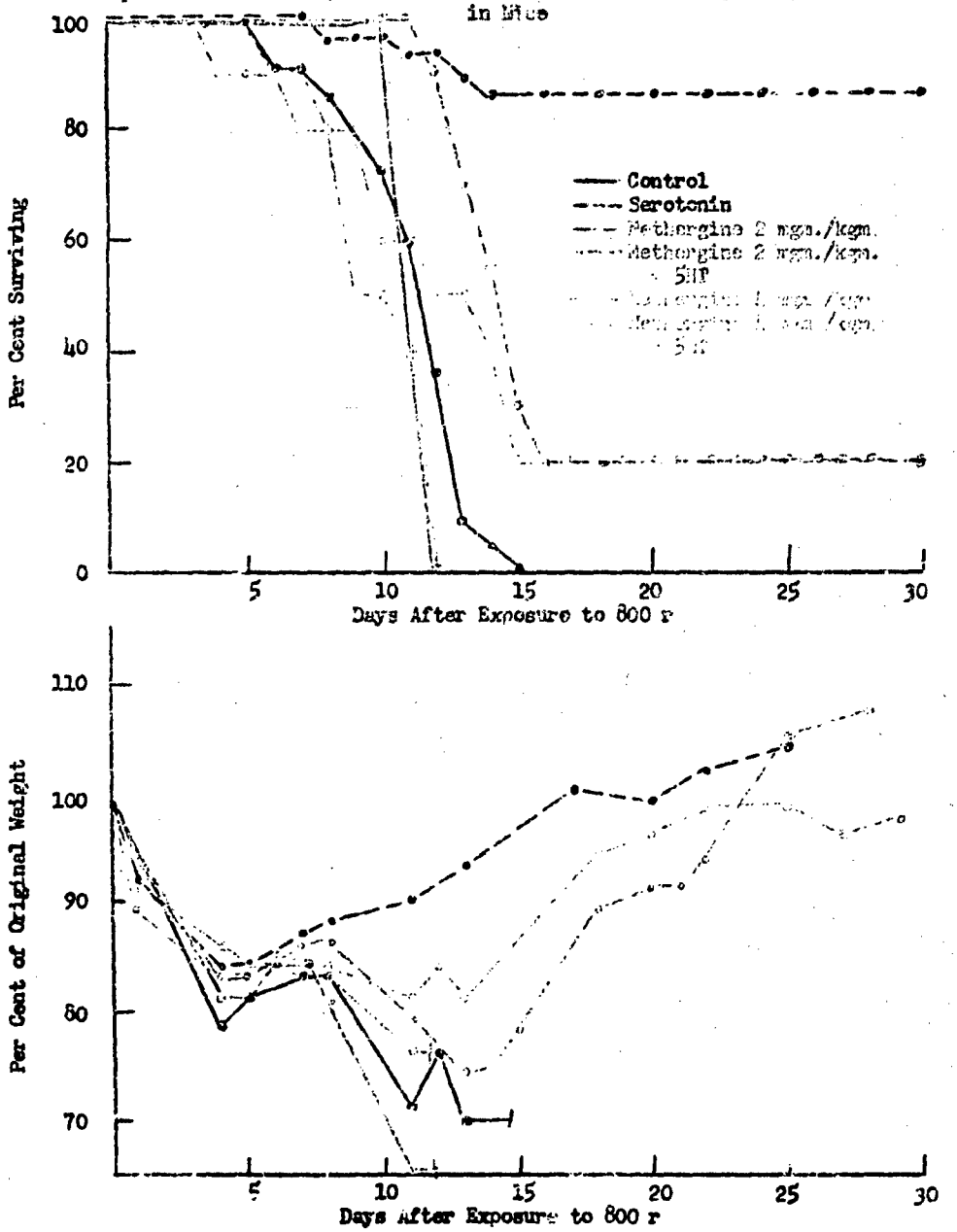


Figure 7. Effect of Methyl Ergonovine (Methergine) on the Radio-protective Activity of Serotonin Creatinine Sulfate (ASP-1) in Mice



at a dose of 50 mgm./kgm. but not at the lower dose of 10 mgm./kgm. Methylergonovine decreased the 30-day survivors from 87% in the serotonin-treated animals to approximately 20% at a dose of either 2 or 4 mgm./kgm. The toxicity of the ergot derivative prevented the testing of higher doses.

The influence of serotonin on the mortality response of mice exposed to varying doses of whole body x-irradiation. Although we have demonstrated that serotonin has a marked protective effect against the mortality and weight loss seen in mice exposed to 800 r of whole body x-irradiation, little information is available concerning the magnitude of the protective effect of this compound at other radiation dosage levels. It was of interest, therefore, to obtain information on the dose-response relationships of x-rayed mice treated with serotonin prior to the radiation exposure. For these studies male and female mice were exposed to doses of whole body x-irradiation ranging from 450 r to 1300 r and comparable groups of mice treated with 90 mgm./kg. of serotonin were exposed to single whole body x-ray doses of 750 r to 1300 r. Groups of eight mice were irradiated at each of the dosage levels investigated and the studies were replicated so that a total of 32 or 64 animals were available at each of the dosage levels. Mortality and weight change observations were made daily until all of the mice in each of the groups were dead. The mortality at thirty days after the irradiation exposure is shown for each of the groups in Table 2. Using the method of Litchfield and Wilcoxon (6) probit mortality regression lines were obtained and are shown in Figure 8. It is evident that the regression line for the serotonin-treated mice is parallel with that of the control animals (slope 1.13 as compared with 1.12) and that the serotonin administration reduces the effective radiation dosage by about 38% throughout the range tested. The LD_{50} of the serotonin-treated mice was 880 r in contrast to a value of 542 r for the LD_{50} of the untreated animals.

In addition to comparing the LD_{50} values for the control and serotonin-treated mice, the median survival time (ST_{50}) of the animals receiving the various doses of x-irradiation was also determined. The method of Litchfield (7) was used for estimating the ST_{50} and an example of the probability survival plot for one of the radiation doses is shown in Figure 9. Similar plots were made for the animals given each of the radiation dosage levels and the ST_{50} values obtained in this manner were then graphed against the log of the radiation dose administered. The results of these studies are shown in Figure 10. It can be seen that the response is linear within the dosage range employed for both the control and serotonin-treated groups but that the protective effect is greater when the lower dosage levels are employed. At a dosage level of about 1300 r of x-irradiation, the median survival time of the serotonin-treated mice is not significantly different from that of the control irradiated animals.

Discussion

The results of the present study, which demonstrate that the protective effects of serotonin against radiation injury in mice can be markedly reduced by the administration of several serotonin antagonists, together with similar findings in rats (5) suggest that the radioprotective activity of this compound is related to one of its several pharmacological activities. Our previous finding that the radioprotective activity of serotonin can be eliminated or markedly reduced in mice by high oxygen pressure during irradiation exposure

TABLE 2

EFFECT OF SEROTONIN ON THE MORTALITY OF MICE EXPOSED TO SINGLE DOSES OF WHOLE BODY X-IRRADIATION

Dose (r)	Number Surviving at 30 Days		Per Cent Surviving at 30 Days	
	Males	Females	Males	Females
Controls				
450	16/16	.....	100	.....
500	13/16	11/16	81.3	68.8
550	11/32	19/32	34.4	59.4
600	1/32	10/32	3.1	31.2
650	0/32	2/16	0	12.5
700	0/32	2/32	0	6.2
750	0/32	0/32	0	0
Serotonin				
750	25/29	12/14	86.2	85.8
800	31/35	11/16	88.5	68.8
850	15/28	21/31	53.5	67.8
900	11/29	21/31	38.0	67.8
950	4/14	1/16	28.5	6.2
1000	8/32	8/29	25.0	27.6
1050	0/32	0/32	0	0

Figure 8

Effect of Serotonin on the Mortality (30-Day) of Mice Exposed to Various Doses of Whole Body X-irradiation

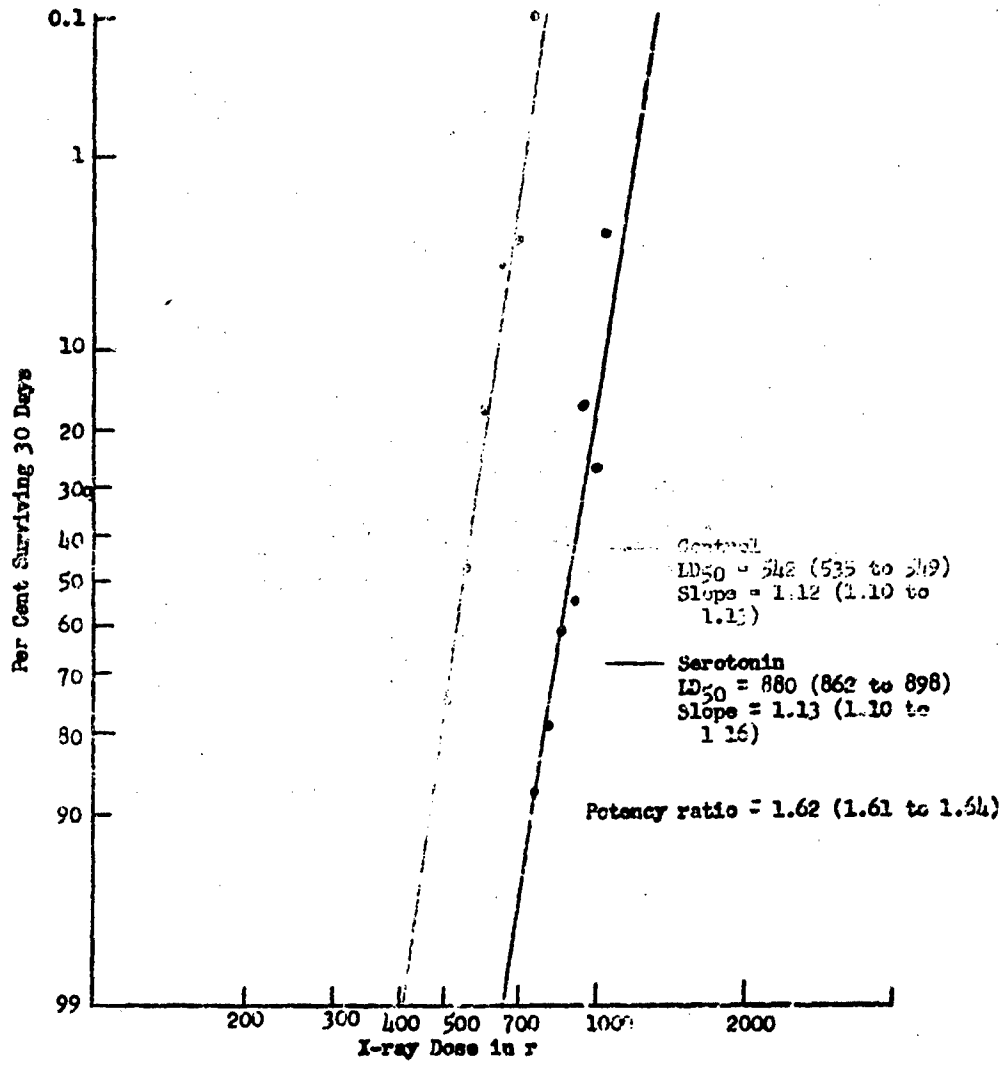


Figure 9

Method of Obtaining EST_{50} Values

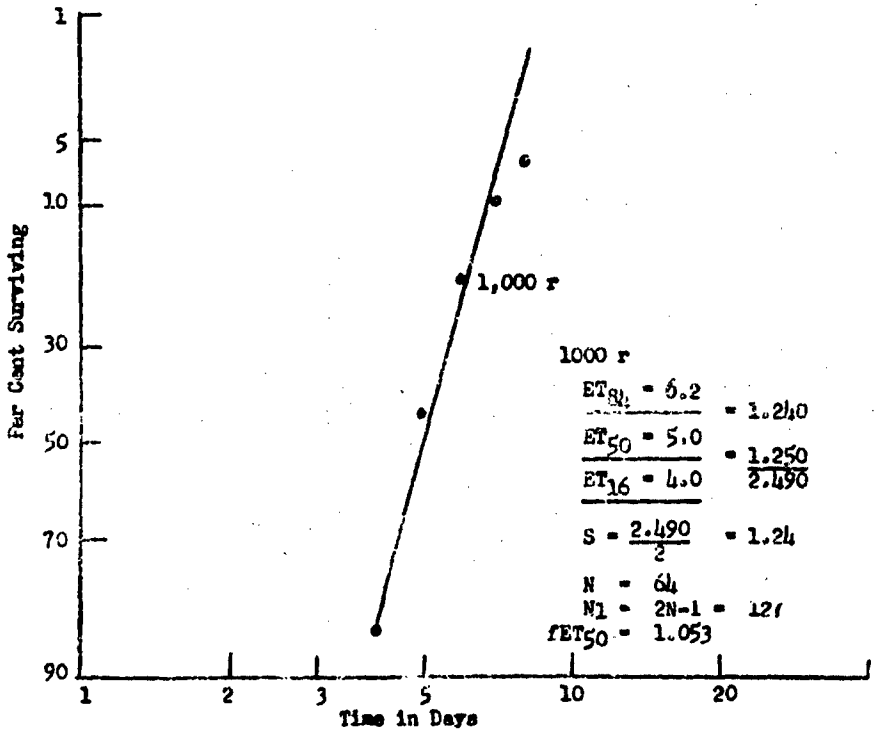
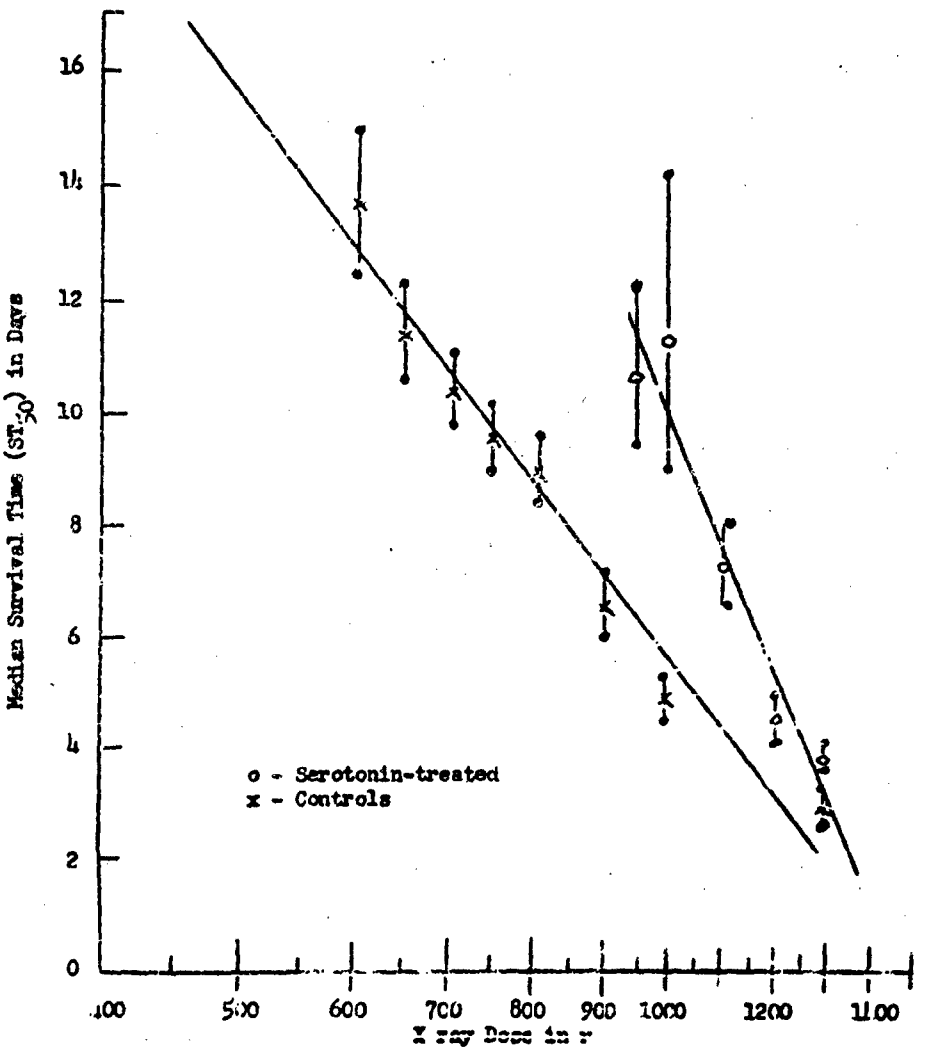


Figure 10

Effect of Pre-treatment with Serotonin on the Median Survival Time (ST_{50}) of Mice Exposed to Varying Doses of Whole Body X-irradiation



and the fact that a similar finding has been obtained in rats (5) further indicate that the pharmacologic effect responsible for the protective activity may be associated with anoxia. Cater and Weiss (8) have demonstrated a reduction in the oxygen tension of the spleen, bone marrow and testis of mice during hypothermia and have suggested that the anoxia thus produced may be responsible for the radioprotective effects of chilling. Since serotonin produces a marked hypothermia, it was of interest to determine whether this effect could be correlated with the radioprotective activity. The results of the present studies demonstrate that even though the rectal temperature of serotonin-treated mice can be reduced by chilling, there is little difference in the 30-day survival of mice kept at reduced or elevated environmental temperatures prior to and during the irradiation exposure. Since prior chilling did not enhance the radioprotective effect in the serotonin-treated animals and since warming the mice did not prevent the radioprotective activity of serotonin, it would appear that the ability of serotonin to produce hypothermia is not a primary factor in its radioprotective activity. Supporting evidence for this conclusion was obtained in the studies designed to determine the most effective time for administering the serotonin to obtain maximal radioprotective activity. Measurements of the rectal temperatures in these mice indicated that the maximal protective effect of serotonin occurred considerably before the period of maximum hypothermia.

Serotonin has been shown to exhibit bronchoconstrictor effects and also vasoconstrictor effects in the pulmonary circulation of dogs, cats and guinea pigs at dosage levels in the order of those employed for these studies (9). These pulmonary effects can be antagonized by lysergic acid diethylamide derivatives. Although these pulmonary effects might contribute to the serotonin produced anoxia, it does not appear likely that they would constitute an adequate means of producing sufficient anoxia for radioprotective activity. If serotonin causes radioprotective effects through the production of anoxia, it would appear more likely that this is due to vasoconstrictor effects in specific radiosensitive tissues or to the production of a cellular anoxia through interference with intermediary metabolism. Serotonin has a wide variety of pressor and depressor effects in different species and many of these can be influenced by the use of serotonin antagonists (9). The possibility that serotonin produces its radioprotective effect by an action on intermediary metabolism has received some support in the work of Bernheim et al. (10) who have shown that serotonin is a powerful inhibitor of the formation of lipid peroxides in vitro which they feel may be involved in the production of radiation effects in vivo (11). Thus, although it is evident that the role of anoxia in the radioprotective activity of serotonin needs clarification, the results of the present studies support the hypothesis that anoxia is involved in the protective action of this compound and further studies are in progress to obtain more detailed information on the precise mechanism involved.

Summary

1. Pre-irradiation administration of serotonin creatinine sulfate is most effective in preventing radiation lethality and weight loss when the drug is given at 10 to 30 minutes prior to the radiation exposure. Maximal

temperature lowering following the administration of serotonin to mice occurs later (one hour or more).

2. Post-irradiation administration of serotonin is ineffective in preventing radiation lethality or weight loss in x-irradiated mice.

3. The ability of serotonin to lower the rectal temperature of mice exposed to a decreased environmental temperature does not appear to be involved in the radioprotective effect of this compound. It was not possible to reverse the radioprotective activity of serotonin by increasing the environmental temperature of the x-irradiated animals either during or prior to the radiation exposure.

4. The radioprotective activity of serotonin against lethality and weight loss in x-rayed mice can be markedly reduced by the prior administration of serotonin antagonists such as the lysergic acid diethylamide derivatives, methyl ergonovine and an experimental antagonist SA-97.

5. Calculation of the dose response relationships between the 30-day survival and the radiation dosage revealed that the serotonin administration reduced the effective radiation exposure by about 40% within the dosage range of 400 r to 1100 r of whole body x-irradiation.

6. Probit mortality-plots of the median survival time of serotonin-treated and control irradiated animals demonstrated that the radioprotective effect of this compound is more pronounced when lower doses of radiation are employed and that at a dosage level of about 1300 r, there is no significant difference in the ST_{50} of the serotonin-treated and control groups of mice.

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PHARMACOLOGICAL AND TOXICOLOGICAL COMPOUNDS AS PROTECTIVE OR THERAPEUTIC AGENTS AGAINST RADIATION INJURY IN EXPERIMENTAL ANIMALS

III. Acid-catalysed Reactions of Aziridines with Thiourea and Its Derivatives

Stanley J. Brois and Gerhard L. Closs

This report concerns: Further studies on the kinetic and synthetic aspects of the acid-catalysed reactions of aziridines with thiourea and its analogs.

Immediate or ultimate application of the results: The role of steric and electronic factors on the SN2 reactivity of 2,2,3-trimethylaziridine has been elucidated employing a spectrophotometric technique. The successful application of our new aziridine synthesis to a spectrum of proto-type tetrasubstituted olefins, both aliphatic and alicyclic, has demonstrated the general utility of this preparative method for procuring 2,2,3,3-tetraalkylaziridines in very good yields. Preliminary synthetic work encompassing the base-catalysed reaction of N-chloro-t-butylamine indicates that the Stieglitz rearrangement also occurs in a purely aliphatic system.

* * * * * * * * *

Consistent with the second order kinetics previously observed (1) in the reactions of aziridines, the present rate study on the ring opening of 2,2,3-trimethylaziridine with thiourea similarly points to an SN2 mechanism for this process. It is noteworthy that on a statistical basis, the trimethyl analog undergoes ring cleavage about twice as fast as trans-2,3-dimethylaziridine and half as fast as the cis-2,3-dimethylaziridine isomer. The intermediate reactivity of trimethyl-substituted imine could be interpreted as a compromise between two opposing factors, namely, an accelerating factor arising from a steric interaction between the cis-oriented methyl groups (eclipsing effect) and a decelerating factor resulting from the ring stabilizing effect of the three methyl groups (Thorpe-Ingold effect). The excellent straight lines obtained when the present rate data were plotted in accordance with second-order laws indicate that the concurrent methanolysis reaction is insignificantly slow.

In a previous report (1), we had described a new synthesis of 2,2,3,3-tetramethylaziridine. This novel synthetic method consisted of the chloronitrosation of tetramethylethylene, the $SnCl_2$:concentrated HCl reduction of the nitroso chloride to the chloroamine, and cyclization of the latter to the imine, ring closure being effected with alkali. In order to demonstrate the general applicability of this novel and convenient synthetic route to tetraalkylaziridines, a series of five fully substituted olefins, both aliphatic and alicyclic, were prepared and processed in the manner described above. In all cases, excellent results were obtained.

In exploring the possibility of generating an intermediate univalent nitrogen species, which might undergo an addition reaction with olefins, a series of base-catalyzed reactions of N-chloro-t-butylamine were investigated. In preliminary work, we have observed that the potassium t-butoxide-catalyzed reaction of N-chloro-t-butylamine at 10, 35 and 55° in the presence of an inert solvent, did not afford an insertion product, viz., 2,2-dimethylaziridine, nor an addition product when cyclohexene was employed as solvent. The isolation of methylamine (as the picrate derivative) in 31% yield reveals the intrusion of a Stieglitz rearrangement (2) in this reaction. In contrast with the monochloroamine, N,N-dichloro-t-butylamine under the same reaction conditions afforded isobutylene as the only detectable reaction product.

Methods and Materials. The following starting materials were employed in the synthesis described in the Experimental section of the report: 2-methylcyclohexanone, 2-methylcyclopentanone, acetone, 2-bromobutane, 2-bromopentane, methyl bromide, magnesium, 1,2-dimethylcyclohexene, 1,2-dimethylcyclopentene, 2,3-dimethyl-2-pentene, 2,3-dimethyl-2-hexene, t-butanol, t-butylamine, potassium metal, and sodium hypochlorite solution ("Clorox").

Kinetic measurements. The Beckman ultraviolet spectrophotometer, model DU, was employed to investigate the kinetics of the SN2 reaction of thiourea with a series of substituted aziridines.

Melting points. Melting points were determined with a Fisher-Johns apparatus and are not corrected.

Absorption spectra. The infrared spectra were determined with a Perkin-Elmer Model 21 recording infrared spectrophotometer. Sample compounds were generally analyzed as pure liquids in a 0.025 mm. absorption cell.

Nuclear magnetic resonance (NMR) spectra. The spectra were run at room temperature employing the Varian Model A-60D High Resolution Spectrometer operating at 40 megacycles per second. A spinning 5 mm. pyrex glass reference tube with a coaxially, precision-fitted sample tube (Zimmerman tube) was utilized in effecting shift measurements, displacements being determined by the "side band" technique with a calibrated Hewlett-Packard 200J audio oscillator. All measurements of peak positions in cps are relative to the external annular benzene reference. Pure liquid samples were used in most cases.

Vapor phase chromatography (VPC) analysis. Product analysis was accomplished by means of VPC using a column of tricresyl phosphate on firebrick with helium gas as the eluting agent. In this work, a Fisher-Gulf Partitioner, Model 300 equipped with a low current thermistor-type thermal conductivity cell and automatic integrator system was employed.

Experimental

Synthesis of olefins. The four tetra-alkyl-substituted olefins employed in the present study were prepared by the classical method of Chavanne (3). It consists essentially of the condensation of a suitable Grignard reagent and ketone, and successive dehydration with an appropriate dehydrating reagent, usually iodine. This procedure will be described in full detail in the preparation of 2,3-dimethyl-2-pentene. In the other cases, only specific details will be presented.

2,3-Dimethyl-2-pentene. To a solution of the Grignard reagent (under a nitrogen atmosphere) prepared from 7.3 moles of 2-bromobutane and 7.3 moles (177 g.) of magnesium metal in 2 liters of anhydrous ether contained in a 5-liter three-necked flask fitted with reflux condenser, dropping funnel and precision stirrer, was added with vigorous stirring, 550 ml. of dry acetone in an equal volume of ether. During the course of the addition, maximum reflux was maintained. After complete addition of the acetone, reflux was continued for two hours and the mixture was then decomposed by the cautious addition of a saturated ammonium chloride solution. The ether layer was separated, washed with water and dried over sodium sulfate. Two distillations gave a product in 37% yield, b.p. 128-131° (4). The resulting tertiary alcohol was subsequently dehydrated with a catalytic amount of iodine. At 100° dehydration began and the temperature was adjusted so that the olefin and water distilled over slowly. The olefin was washed with a sodium thiosulfate solution to remove any free iodine and dried over calcium chloride. After refluxing the olefin with sodium metal for a half hour, the alkene was twice distilled through a 24" Podbielniak column. The purified olefin, obtained in 57% yield, boiled at 93-95° (5). The VPC analysis of this derivative through a 12' column containing 30% tricresyl phosphate on firebrick at 62° and a He flow rate of 95 ml./minute indicated a purity of 83%.

2,3-Dimethyl-2-hexene. In similar fashion the Grignard reagent of 2-bromopentane (3.3 mole) was prepared in 1 liter of anhydrous ether in a nitrogen atmosphere. After addition of 3.3 moles of acetone under maximum reflux conditions, the reaction mixture was decomposed with concentrated ammonium chloride solution. Fractionation of the dried ether extract afforded a 40% yield of the carbinol, which was not isolated but directly processed with iodine. Distillation of the dehydration product over sodium metal through a 24" Podbielniak column gave a fraction, b.p. 115-119° (5) in 53% yield. The VPC analysis of this olefin through a 12' column containing 30% tricresyl phosphate on firebrick at 82° and a He flow rate of 95 ml./minute indicated a purity of 89%.

1,2-Dimethylcyclopentene. One mole of 2-methylcyclopentanone, b.p. 140-142°, was converted to the corresponding carbinol by the action of the methyl Grignard reagent. The dried, fractionated product boiled at 60-66° (6) at 20 mm. The olefin was procured in 85% yield by refluxing the alcohol with a trace of iodine for two hours and collecting the products by distillation. The fraction of cycloalkene boiling at 103-105° (5) was collected and subjected to VPC analysis which indicated that the olefin was 91% pure.

1,2-Dimethylcyclohexene. The methyl Grignard reagent was prepared in the usual manner and reacted with 6.3 moles of 2-methyl-cyclohexanone in two liters of ether. The resulting carbinol, obtained in 89% yield, was distilled, b.p. 106-109° (100 mm.), and then dehydrated with a catalytic amount of iodine. Dehydration began at 100° and the temperature was so adjusted that the olefin distilled over gradually. After washing the cycloalkene with thiosulfate solution and drying with magnesium sulfate, it was refluxed with sodium metal for an hour. Distillation afforded 503 grams (72.5% yield) of product boiling at 135-137° (5) at 755 mm. The purity of the 1,2-dimethylcyclohexene was assessed by VPC analysis and found to be 70%.

Three-Step Aziridine Synthesis

1. Chloronitrosation. The following general procedure for the addition of nitrosyl chloride to olefins appeared most suitable. A well-stirred solution of 0.1 mole of the olefin in 200 ml. of absolute methanol cooled in a dry ice-acetone bath, was gradually saturated with the stoichiometric quantity of nitrosyl chloride gas. In the course of the addition, the colorless reaction mixture became intensely blue and the nitroso chloride derivative partially separated from solution. After the addition of the gaseous nitrosyl chloride was complete, the cooling bath was removed and the mixture was rapidly stirred for one hour. Cautiously, the deep-blue colored solution was poured into 500 ml. of ice water. The nitroso chloride immediately separated as a blue solid which was washed twice with water, filtered, and air-dried. Quantitative yields were obtained in all cases. For illustration, the nitroso chloride of 1,2-dimethylcyclohexene, m.p. 78-79°, was characterized. Anal. calcd. for $C_8H_{14}NOCl$: Cl, 20.19%. Found: Cl, 20.29%.

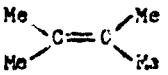
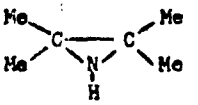
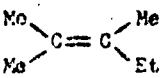
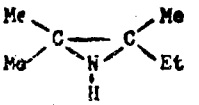
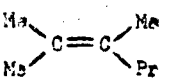
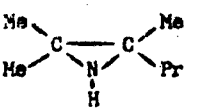
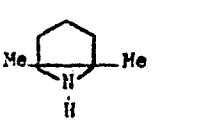
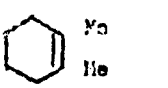
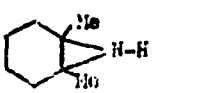
2. Reduction by $SnCl_2$:HCl combination. After conducting the reduction reaction under a variety of experimental conditions, the following procedure appeared to give the best results. A solution of 90 grams (0.4 mole) of stannous chloride dihydrate (Reagent grade, Fisher certified, ACS) in 120 ml. of concentrated hydrochloric acid (12 N) in a 500 ml. Erlenmeyer flask was mechanically stirred in an ice bath until a temperature of 5° was obtained. At this point, the ice bath was removed and a 0.1 mole of the nitroso chloride derivative was added in one portion (addition in several portions produces lower yields). In general, the reaction temperature rose to 50-70° in the course of an hour, accompanied by the gradual disappearance of the blue nitroso chloride. Within two hours the temperature began to decrease and the clear solution was allowed to cool to room temperature.

3. Cyclization. The cooled reaction mixture derived from the above reduction was carefully added in a dropwise manner to a rapidly stirred ice-cooled solution of 2 moles of sodium hydroxide in a liter of water. The basified mixture was transferred into a 3-liter three-necked flask equipped with thermometer, stirrer, and condenser set for downward distillation into an ice-cooled receiver containing a few sodium hydroxide pellets. Steam distillation of the reaction mixture was continued until the distillate indicated no basic reaction to litmus. The strongly basified distillate was extracted twice with ether and the resulting ethereal solution was dried over sodium. After stripping off the ether, the imine derivative was refluxed with sodium metal for an hour and subsequently twice distilled through a 24" Podbielniak column. The yields, physical constants, and elemental analysis for the aziridine analogs synthesized are presented in Tables 1 and 2.

Characterization of 9-chloro-10-decalinamine. The elemental analysis of the 9-chloro-10-decalinamine HCl derived from the hydrochloric acid-catalyzed ring opening of the alleged 9,10-iminodecalin earlier reported (1) lends support to the proposed imine structure for this compound. Anal. calcd. for $C_{10}H_{19}NCl_2$: Cl, 31.63%. Found: Cl, 31.23%. A duplicate experiment to prepare the 9,10-iminodecalin from the corresponding olefin, 9,10-octalin, was undertaken in this present research. Distillation of the alleged aziridine afforded 12.2 grams of a fraction boiling at 84-85° at 5 mm. The infrared spectra of this product disclosed a band at 3245 cm^{-1} as observed previously, and may be ascribed

TABLE 1

2,2,3,3-TETRAALKYLAZIRIDINES

Starting Material Olefin	Product Imine	Yield[a] %	B.P., (mm.) °C	n_D^{20}
		79	104-104.5 (744)	1.4220
		71	129-129.5 (751)	1.4312
		84	150-150.5 (747)	1.4339
		73	134-135 (752)	1.4550
		76	165-165.5 (750)	1.4665

[a]Yields are calculated on the basis of pure olefin employed in a 0.1 mole reaction.

TABLE 2

ELEMENTAL ANALYSIS OF 2,2,3,3-TETRAALKYL-AZIRIDINES

Compound	Calc'd.		Found	
	C	H	C	H
2,2,3,3-Tetramethyl-aziridine	72.66	13.21	72.96	13.47
2,2,3-Trimethyl-3-ethylaziridine	74.27	13.36	74.47	13.35
2,2,3-Trimethyl-3-propylaziridine	75.52	13.47	75.46	13.67
1,2-Dimethylcyclo-hexenimine	76.73	12.07	76.52	11.87
1,2-Dimethylcyclo-pentenimine	75.61	11.78	75.31	11.76

to the N-H stretch frequency. The spectral and analytical data present convincing arguments in favor of the proposed structure for this derivative.

Preparation of N-chloro- and N,N-dichloro-t-butylamine. An adaptation of the procedure reported by Klages (7) was employed for the synthesis of both chloroamines. To a solution of 73.1 grams (1 mole) of t-butylamine in 200 ml. of ice water contained in a 5-liter flask equipped with stirrer, thermometer, and dropping funnel, and cooled in an ice-bath was added in a dropwise manner 1.42 kgm. of 5.25% sodium hypochlorite solution ("Clorox") at such a rate as to maintain the temperature below 10°. After the addition was complete, the ice bath was removed and the reaction mixture was stirred for one hour. After extracting the latter with three 100 ml. aliquots of ether, the combined aliquots were dried over calcium chloride. The ether was stripped off, and distillation of the residue at 70 mm. afforded a colorless liquid (63% yield) boiling at 39-40°. The monochloroamine is extremely lacrimatory and possesses a stifling odor. The synthesis of the N,N-dichloro-t-butylamine is accomplished in the exact same manner except that two equivalents of the sodium hypochlorite are employed. The characterization of the N,N-dichloro analog is pending.

Synthesis of potassium t-butoxide. The procedure used is essentially that reported by von Doering and Hoffmann (8). The reaction of potassium metal, 1.5 moles, and 2 liters of anhydrous t-butanol was carried out in a 3-liter three-necked flask equipped with reflux condenser (mounted with a drying tube) with rapid stirring at reflux temperature. When all of the potassium metal had reacted, the stirred solution was concentrated by distillation and finally vacuum dried at 160° for five hours. The dry potassium t-butoxide was assayed with standard hydrochloric acid (1 N) and stored in a desiccator.

The Base-catalyzed Reaction of N-Chloro-t-butylamine

1. Reaction in n-pentane at 35°. To a mixture of 22.5 grams (0.2 moles) of $KOCMe_3$ in 50 ml. of n-pentane in a 500 ml. three-necked flask fitted with thermometer, reflux condenser (mounted with a drying tube), and dropping funnel, was added in a dropwise manner, 10.8 grams (0.1 mole) of the N-chloroamine in 25 ml. of n-pentane at such a rate as to maintain mild refluxing. After the addition was complete, stirring was continued for fifteen minutes. The condenser was set for downward distillation and the volatile products and solvent were distilled out of the reaction flask and subsequently fractionated through a 24" tantalum spiral column. Five fractions, b.p. 32-35° (5.2 g.), 35-36° (39.5 g.), 38-44° (3.2 g.), 69-81° (3.3 g.), and 81-83° (4.3 g.) were collected, labelled and submitted for infrared analysis. For comparison purposes, the spectra for t-butanol, t-butylamine, and 2,2-dimethylaziridine were also taken.

2. Reaction in n-octane at 55°. In the same manner as described above, the reaction of 0.2 mole of t-butoxide with 0.1 mole of N-chloroamine was carried out at 55° in octane. The anhydrous reaction mixture was fractionated and five fractions, b.p. 41-44° (2.8 g.), 68-72° (2.5 g.), 73-80° (5.5 g.), 79-81° (7.8 g.), and 81-83° (3.5 g.) were obtained, and spectrally analyzed.

3. In cyclohexene at 10° and 35°. In an analogous fashion, the reaction of 0.8 mole of t-butoxide with 0.5 moles of N-chloroamine was conducted in 123

grams of cyclohexene at 10° and 35°. In these experiments our interests were focused on the addition product between the olefin and an intermediary $Me_3C\text{-}N$: species. The N-t-butyl aziridine analog, if present, would appear in the higher boiling fraction namely, above 100°. Distillation of the reaction mixture did not afford any fractions boiling above 91°.

4. In pentane at 10°. The reaction of 0.22 mole of t-butoxide with the N-chloroamine (0.1 mole) in 50 ml. of pentane was carried out as described above, except that the reaction mixture was hydrolyzed with an excess of 2 N hydrochloric acid. The organic layer was dried, fractionated, and spectrally analyzed. The aqueous layer was fractionated (lower boiling cuts were spectrally analyzed) and the residue was subsequently evaporated to dryness on a Rinco evaporator. In the event the N-chloro-t-butylamine had undergone a Stieglitz rearrangement to N-isopropylidene methylamine, the methylamine produced under the acid hydrolysis conditions would be contained in the residue as the hydrochloride salt. With this in mind, the residue was treated with concentrated alkali solution in a flask connected to a 24" tantalum spiral distilling column. In this experimental design, only the volatile methylamine, b.p. -6°, would be able to bypass the condenser at the head of the column. The volatile product was directed into an ether solution of picric acid contained in the receiver flask. A significant quantity of yellow precipitate formed in the course of the reaction. After the evolution of the gas ceased, the product was filtered, air-dried, and weighed. The methylamine picrate derivative (31% yield) indicated a m.p. of 213-214° after two recrystallizations from ethanol. The m.p. reported for the methylamine picrate is 215° (9). Additional work on this problem is in progress.

Preparation of Raney nickel catalyst. The procedure reported by Mozingo (10) was employed in the following synthesis. A solution of 380 grams of sodium hydroxide in 1.5 liters of distilled water contained in a 4-liter beaker equipped with an efficient stirrer was cooled in an ice bath to 10° and 3 kgm. of $NiAl_2$ alloy was added at such a rate as to maintain the reaction temperature below 25°. When all of the alloy was added, stirring was ceased and the contents were allowed to come to room temperature. After hydrogen evolution subsided, the reaction mixture was allowed to remain on the steam bath until hydrogen evolution ceased. The liquid was decanted, fresh distilled water was added and the nickel was suspended in the aqueous solution. Once the nickel settled, the solution was again decanted and the residue is transferred to a 2-liter beaker. A solution of 50 grams of NaOH in 500 ml. of distilled water is added and the catalyst is suspended, allowed to settle and the alkali solution is decanted. The nickel is washed in this manner about thirty times (until the washings are neutral to litmus) and subsequently stored under distilled water.

Kinetics

Reagents. Methanol (Baker and Adamson absolute methanol) was employed as solvent in all cases. Reagent grade chemicals which included thiourea (Matheson Company, m.p. 176-173°) and sulfuric acid were employed without further purification. The AET salt. α, β, β-trimethyl AET SO_4 was recrystallized three times from aqueous ethanolic sulfuric acid. The 2,2,3-trimethylaziridine was synthesized via the Wenker method, dried over sodium metal and distilled immediately before a series of kinetic runs.

Spectrophotometric procedure in kinetic runs. All apparatus and glassware used in the preparation of solutions employed in the rate experiments were treated with cleaning solution rinsed several times with tap water and six times with distilled water and finally dried in an oven maintained at 110°.

Spectrophotometric measurements were made with a Beckman spectrophotometer, model DU, equipped with a water-cooled hydrogen source. The absorbance readings for thiourea were taken at the maximum sensitivity setting for the instrument.

Methanolic solutions of thiourea were prepared in the following manner: the required amount of thiourea was weighed out and transferred to a clean, dry 25 ml. volumetric flask. About 10 ml. of methanol were added to the flask which was then immersed in an ice bath. To the cooled solution was added very gradually a measured volume of concentrated sulfuric acid. The flask contents were diluted to the mark. The methanolic solutions of the imine were prepared by adding a slight excess of the aziridine with a calibrated syringe to a tared 10 ml. volumetric flask. Evaporation of the slight excess of imine was accomplished by permitting a mild jet of dry nitrogen to flow over the orifice of the flask. Within a few minutes, the exact weight of the imine was obtained and flask contents were immediately diluted with methanol to the mark.

A 100 ml. volumetric flask containing methanol was equilibrated in a water thermostat maintained at 24.55° ± .04°. The solvent volume was adjusted to the mark and then an appropriate volume of methanol was withdrawn with a transfer pipette maintained at reaction temperature. The thiourea solution was admitted into the flask in the same manner. Allowing ten minutes for temperature equilibration, the reaction was initiated by carefully pipetting the imine solution into the flask containing the thiourea to give a final volume of 100 ml. The half-way time of the imine delivery was considered as the starting time for the reaction. The time required for thorough mixing of the flask contents was about 10 seconds. Aliquots of the reaction mixture were quickly withdrawn at predetermined times. A calibrated transfer pipette was brought quickly to the mark and rapidly discharged into a volumetric flask containing methanol at 20°. In all cases the dilution range used was effective in quenching the reaction as indicated by the fact that the absorbance of the diluted sample remained constant over a 4-hour period. The elapsed time for sampling was less than fifteen seconds. A small volume of the quenched solution was transferred into a clean silica cell which was rinsed about 8-10 times with the quenched solution and previously corrected against a reference cell. The concentration of the quenched solution was predetermined in order to effect measurements in the most favorable transmittancy range. The sample cell and the reference cell containing solvent were carefully oriented in the cell holder and then inserted into the cell compartment of the DU spectrophotometer. The temperature of the cell compartment was maintained at 20° by circulating water from a thermostated bath through thermospacers arranged on each side of the cell compartment. Absorbance readings were generally taken at 10-15 minute intervals until 50% completion of reaction.

Ultraviolet absorption characteristics of α,β,β-trimethyl AET SO_4. Triplicate experiments to determine the molar absorptivity of α,β,β-trimethyl AET SO_4 (ϵ = 1300), revealed that this AET derivative conformed strictly to Beer's law for the range of concentrations employed in these experiments (ca. 10^{-3} to 10^{-5} M), the absorption at 241 mμ being employed. Graphical treatment

of absorbance vs. concentration for this AET salt is depicted in Figure 1. Absorbance vs. concentration plots indicate no interaction between product and reactant and that the absorbancies of thiourea and its S-alkylated analog are strictly additive. In observing the disappearance of thiourea with time, the apparent absorbance was converted to the true absorbance by correcting for the contribution to the optical density by the AET salt.

Kinetics of the thiourea-imine reaction. The results of the kinetic work have been treated with the assumption that the imine-thiourea interaction was second order in character. When the rate data obtained from the spectrophotometric measurements were plotted as a function of

Equations 1 and 2 $\frac{1}{a-x}$ (a=b) or $\log \frac{(a-x)}{(b-x)}$ (a=b)

against time t, where a is the molar concentration of thiourea, b is the molar concentration of the imine, and x is the number of moles of thiourea that has reacted in time t, straight lines were obtain in all instances indicative of strict second order kinetics. The specific rate constants were determined graphically and by the appropriate bimolecular equations from the data covering about 50% reaction. Figure 2 and Table 3 illustrate the kinetic analysis of the reaction of 2,2,3-trimethylaziridine with thiourea. In general, most rate constants were estimated with a very good degree of precision with the average deviation being in the order of 2-3% for the mean and reproducible to the extent of 1-2%.

Discussion

Earlier work on our new aziridine synthesis culminated in the preparation of the hitherto unknown 2,2,3,3-tetramethylaziridine. In this present research we attempted to demonstrate the general application of this synthetic procedure to both aliphatic and alicyclic tetra-substituted olefins. For this purpose, a series of proto-type alkenes, 2,3-dimethyl-2-pentene, 2,3-dimethyl-2-hexene, 1,2-dimethylcyclopentene and 1,2-dimethylcyclohexene were prepared via the classical method of Chavanne as described in the Experimental section of this report. Good results were obtained in the synthesis of the intermediary carbinols derived from 2-methylcyclohexanone and 2-methylcyclopentanone and the methyl Grignard reagent. On the other hand, the condensation of acetone with 2-bromobutane and 2-bromopentane Grignard reagents gave poor yields of the desired carbinols. The latter results could be ascribed to a significantly competitive reduction reaction and illustrates the desirability of employing the methyl Grignard reagent when optional synthetic pathways exist. The dehydration of the alcohols was accomplished with a catalytic amount of iodine and the resulting olefins were dried, refluxed with sodium metal, and fractionated. Vapor phase chromatography (VPC) was employed to assay the purity of the distilled alkenes. In general, the VPC analysis indicated that the olefins were 70-91% pure (see Experimental).

The chloronitrosation of the alkenes proceeded smoothly and in quantitative yields at acetone-dry ice temperatures. From previous experience, the reduction of the nitroso chlorides was best effected by the addition of the nitroso chlorides in one portion to an excess of stannous chloride:concentrated HCl

Figure 1

Absorbance vs. Concentration Plot for
α, β, δ-Trimethyl AET SO_4

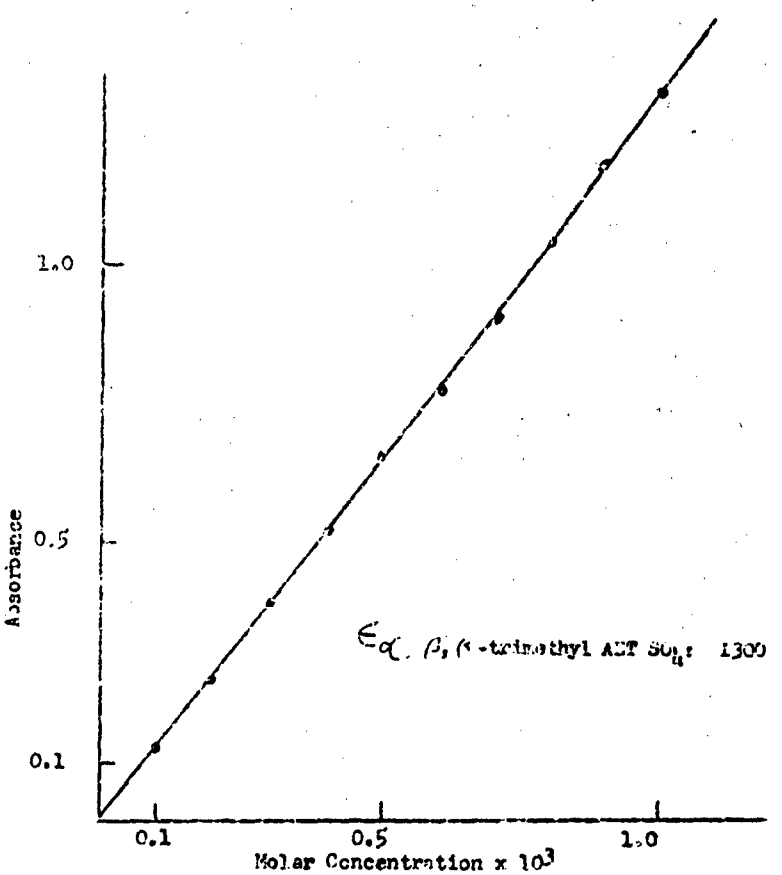


Figure 2

Reaction of 2,2,3-Trimethylaziridine with Thiourea at 21.5° C.

((Figure includes four experimental runs))

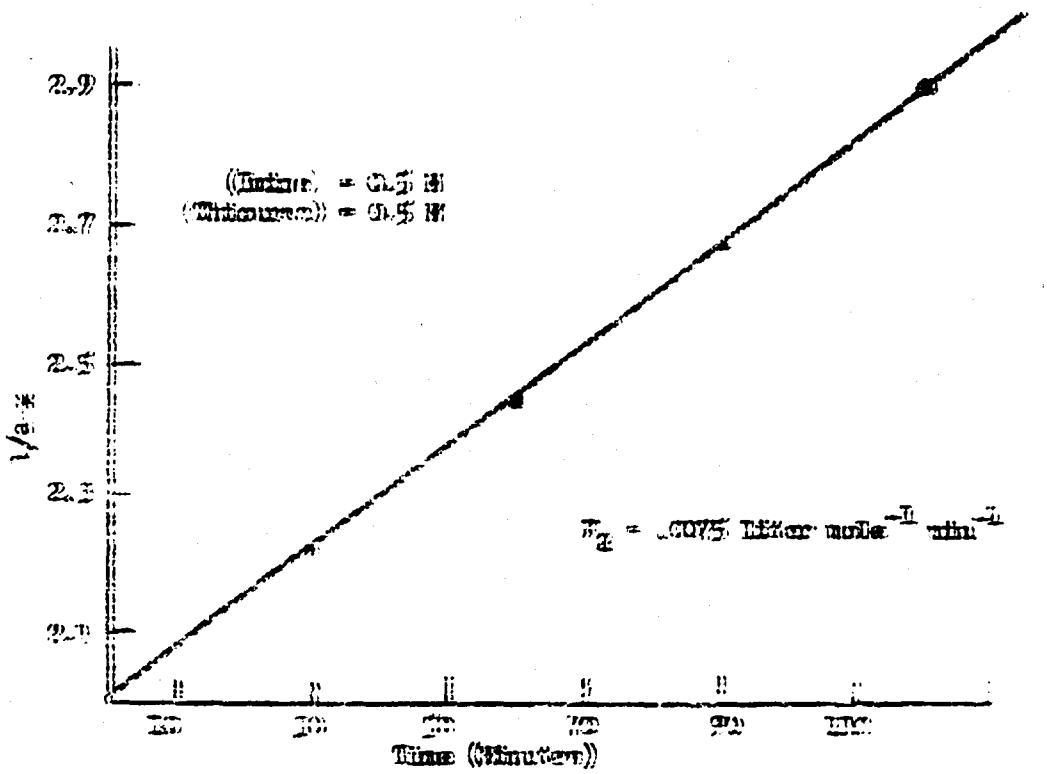


TABLE 3

KINETIC DATA[a] FOR THE REACTION OF THIOUREA WITH 2,2,3-TRIMETHYLAZIRIDINIUM ION IN METHANOL AT 24.55° C.

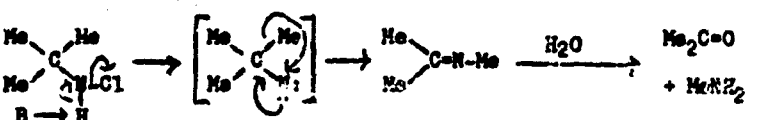
Time (Minutes)	Absorbance (at 241 mμ)	(x)[b]	(a-x)	$2/t(x)/a(a-x)=k_2$ (liter/mole/min.)
0	1.25	0.000	0.500	.....
30	1.14	.050	.450	.0074
60	1.05	.090	.410	.0073
90	0.97	.125	.375	.0074
120	0.90	.155	.345	.0075
			Mean	.0074 ± .0001

[a]The mean value for k_2 obtained from kinetic data when (thiourea) = 0.5 M and (imine) = 0.6 M is .0075 ± .0002 liter $mole^{-1}$ min^{-1}. The final mean value for k_2 computed form the average values in five experimental runs is .0075 ± .0002 liter $mole^{-1}$ min^{-1}.

[b](x) denotes the number of moles of thiourea in each liter reacting in the interval of time t. For these data (x) is the mean derived from four kinetic runs at the concentration range specified.

solution maintaining the reaction temperature at 50-70° (conducting the reduction at ice-bath temperatures tended to diminish the yield of the imines). The base-catalyzed cyclisation reaction was straightforward. The aziridine analogs, which were obtained in 71-84% yields (see Table 1) based on the amount of pure alkene employed, were dried over sodium metal, and twice distilled. The yields, physical constants, and elemental analysis are illustrated in Tables 1 and 2. The infrared spectra of the aliphatic imines, 2,2,3,3-tetramethyl-, 2,2,3-trimethyl-3-ethyl-, and 2,2,3-trimethyl-3-propylaziridine were characterized by an absorption peak at 3200 cm^{-1} arising from the N-H stretching frequency. In the case of the bicyclic imines, 1,2-dimethylcyclopentenimine (1,5-dimethyl-6-azabicycle (3.1.0) hexane) and 1,2-dimethylcyclohexenimine (1,6-dimethyl-7-azabicyclo (4.1.0) heptane), the N-H stretching band was slightly shifted to higher frequency (3225 cm^{-1}) which might be anticipated on the basis of the larger internal strain inherent in these bicyclic systems. In addition, the spectra for these bicyclic analogs are strikingly similar to the infrared spectra reported by Fanta (11) for the lower homologs, namely cyclohexenimine and cyclopentenimine, which also exhibit an N-H band at 3225 cm^{-1}. The NMR spectra of tetramethylaziridine reveals a single resonance peak at 217 cps (relative to benzene as the external reference at 40 megacycles) nicely illustrating the anticipated equivalency of the four methyl groups on the aziridine ring. The NMR spectra for the other aliphatic and bicyclic imines are consistent with expectations. The five imines prepared will be converted to the corresponding AET analogs in conjunction with the kinetic and radioprotection studies being pursued in this laboratory.

Recently, interest has been stimulated in developing a general synthetic approach to aziridines involving the facile addition of a univalent nitrogen species ("nitrene") to an olefin analogous to the reaction of carbenes with alkenes. For this purpose, we chose to explore as a working model the Me_2C-N: moiety which we attempted to generate from the base-catalyzed reaction of N-chloro-t-butylamine. In light of the insertion reaction of the related Me_2C-CH: species which affords 1,1-dimethylcyclopropane, and the rearrangement of dimethylphenylmethyl monochloroamine, $Me_2C(Ph)$-NHCl, to N-isopropylidene aniline (Stieglitz rearrangement), we initially investigated the reaction of N-chloro-t-butylamine with potassium t-butoxide in an inert solvent at various temperatures in the absence of an olefin. In these experiments, which were carried out in either pentane or octane, an exothermic reaction occurred when the N-chloroamine was carefully added to a rapidly stirred excess of t-butoxide. The infrared spectra of the fractionated reaction mixture disclosed the presence of t-butylamine, t-butyl alcohol and solvent (identification was effected by a comparison with reference spectra) and also an absorption band at 1665 cm^{-1} (6.01 u) which appears to be characteristic for the -C=N- stretch frequency observed for imines. This finding suggested that a rearrangement had occurred and may be depicted as follows:



B = potassium t-butoxide

In a subsequent experiment, the hydrolyzed reaction mixture was carefully processed (see Experimental) and methylamine was isolated as the picrate derivative

(b. p. 113-114°) in 31% yield. Spectral analysis of the fractionated organic and aqueous phases revealed absorption bands at 1665 cm^{-1} (imine) and 1710 cm (ketone). These data appear to provide a convincing argument in favor of the formation of a Stieglitz rearrangement product. Confirmatory work is now in progress.

In two subsequent experiments the t-butoxide-catalyzed reaction of the N-chloro-t-butylamine was conducted in cyclohexene at 10° and 55° in order to test the possibility of an addition reaction. Careful fractionation of the reaction mixture gave no evidence of an addition product in either instance. This reaction will be repeated in order to determine whether addition could be effected with a more nucleophilic alkene as tetramethylethylene. It is interesting to note that the base-catalyzed reaction of the dichloroamine, N,N-dichloro-t-butylamine, in contrast to the monochloroamine which rearranged under these conditions, pursued a different course. Fractionation of the reaction mixture afforded a low boiling fraction which disclosed absorption bands at 3050 and 1650 cm^{-1} characteristic for an olefin. VPC analysis of this product revealed the presence of a compound having the same retention time as isobutylene.

The kinetic data compiled in the present investigation demonstrates that the kinetics of the reaction of thiourea with 2,2,3-trimethylaziridine are of the second order. It is noteworthy that the ring opening in the case of the trimethyl analog proceeds twice as fast as trans-2,3-dimethylaziridine and half as fast as the corresponding cis-2,3-dimethyl isomer on a statistical basis. It appears that the rate of SN2 attack on the secondary carbon atom in this instance is governed by two factors, namely an accelerating one arising from steric interaction between the eclipsed methyl groups and a decelerating factor caused by the stabilizing effect of the three methyl groups. The intermediate reactivity of the trimethyl imine could be interpreted as a resultant of these opposing factors. The straight lines obtained when the rate data were plotted according to the conventional rate laws firmly establishes that the concurrent methanolysis reaction of the 2,2,3-trimethylaziridinium ion is very slow.

Summary

Rate measurements effected spectrophotometrically indicate that the reaction of thiourea with the unsymmetrical imine, 2,2,3-trimethylaziridine is second order in character. The electrophilic behavior of 2,2,3-trimethylaziridine relative to the cis and trans 2,3-dimethylaziridine analogs are discussed in terms of steric factors. The application of our new aziridine synthesis to a series of five stereo-type tetraalkyl substituted olefins, both aliphatic and alicyclic, illustrates the versatility of this preparative route to 2,2,3,3-tetraalkylaziridines in good yields. Preliminary studies on the base-catalyzed reactions of N-chloro-t-butylamine and N,N-dichloro-t-butylamine are reported.

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PHARMACOLOGICAL AND TOXICOLOGICAL COMPOUNDS AS PROTECTIVE OR THERAPEUTIC AGENTS AGAINST RADIATION INJURY IN EXPERIMENTAL ANIMALS

IV. Detoxification of 2-Aminoethylisothiuronium Dichloride (AET) in Mice

H. D. Landahl and Andrew T. Kasegawa

This report concerns: In vivo detoxification of AET in normal and hepatectomized mice.

Immediate or ultimate application of results: To obtain information on the detoxification rate of a radiation protective compound and the role of the liver in this function.

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A method is presented for estimation of the detoxification rate of 2-aminoethylisothiuronium dichloride (AET). A study of the detoxification of this drug requires relatively few animals and makes it possible to obtain results without delay. The procedure can be applied where the death endpoint is fairly abrupt and where the time constants of the drug action are within a suitable range of values. The term detoxification is used here to include excretion and conversion to a less toxic compound.

In the case of AET, the accumulated toxic dose was estimated by injecting 100 mgm./kgm. (1% body weight of a solution containing 10 mgm./ml.) at time zero and again at seven minutes. At fifteen minutes and at each 10-minute interval thereafter an amount corresponding to 50 mgm./kgm. was injected (0.5% body weight). The endpoint recorded was the extension of both hind limbs in convulsive movements. Death occurred within a few minutes thereafter with rare exceptions. The accumulated lethal dose was calculated by the somewhat arbitrary assumption that the toxic material is entering linearly, the process being complete in the 10-minute interval. Thus an animal convulsing five minutes after receiving a dose of 50 mgm./kgm. would be assigned an accumulated lethal dose equal to the sum of the amounts injected exclusive of the last one plus 0.5 of the last injection dose.

In order to estimate a detoxification rate, other mice were given 250 mgm./kgm. and allowed to remain at 30° C. for a fixed period of time before being tested for an accumulated lethal dose. Although no normal male or female mice died within three hours from a single dose of 250 mgm./kgm. (2.5% of the body weight), death of partially hepatectomized animals given this dose occurs. Hence throughout this report the 250 mgm./kgm. dose was given in fifteen minutes just as the first 250 mgm./kgm. were given to obtain the accumulated toxic dose. In obtaining the accumulated toxic dose at a fixed time after the conditioning dose; the injection procedure was modified by giving the second

100 mgm./kgm. in two half doses at five and ten minutes instead of a single dose at seven minutes after starting. For the 2-hour and 1-day post partial hepatectomy the first dose was cut in half. The purpose of these variations was to insure that few if any deaths would occur until the time that the injections were spaced ten minutes apart.

Injections at up to twice the rate (e.g., 5-minute intervals instead of 10-minute intervals) increases the accumulated lethal dose by a negligible amount. Using 20-minute instead of 10-minute intervals only slightly increases the value. Using doses of 50 mgm./kgm. every 7.5 minutes throughout also gave about the same accumulated lethal dose. Furthermore, the dose obtained from a freshly neutralized solution was not significantly larger than that obtained using unneutralized solutions. Doubling the concentrations had little or no effect. Water solutions a day old were apparently the same as freshly made solutions whereas neutralized solutions left over night were nearly twice as toxic. As will be discussed later the temperature at which the mice are kept during the detoxification period is important. However, for female mice there was no difference when the mice were kept at 20, 25 or 30° C. while the injections were being made to obtain the accumulated toxic dose.

In order to ascertain the role of the liver in the detoxification process female mice were partially hepatectomized and subsequently treated after allowing various times for regeneration. The earliest time interval used was two hours. Prior to that time the surgical wound was not sufficiently healed to prevent leakage of the drug. The results are given in Table 1. Included also are values for wet liver weights. No differences were found between liver weights with and without the conditioning dose. Values after the ± signs are estimates of the standard deviation of the population. Since an occasional animal will die very early or very late, the highest and lowest values were omitted before obtaining an average for each group in the tables. The numbers in parenthesis are the number of mice used to obtain the values shown as the accumulated lethal doses.

From the data it can be seen that the amount of detoxification is less after hepatectomy but the decrease is not significant. Thus the remaining lobes can handle the compound almost as well as the intact liver. Note, however, that the amount detoxified in three hours is not large and a longer time might reveal differences. The above results are consistent with a model in which the liver acts as a reservoir for the substance, or its toxic product, to the extent of about 25% of the injected dose. Note that at one day the liver weight is up but that there has been no appreciable increase in liver cells by this time.

Male mice, about 80 days old, were used, six in a group, to follow detoxification with time. Two out of eight animals died in the 3-hour partially hepatectomized group. The results of these experiments are shown in Table 2. It can be seen that detoxification is much more rapid in males, detoxification being essentially complete in three hours whereas not much more than 20% occurred in females during the same interval. On the other hand, there is no sex difference in the toxic levels (409 for females versus 420 for males). It can also be seen that the partially hepatectomized male mice showed a marked decrease in detoxification rate for both the 2- and the 3-hour intervals. But there is no appreciable sex difference in the toxic level for the partially hepatectomized mice.

TABLE 1

ACCUMULATED LETHAL DOSE AND THREE-HOUR DETOXIFICATION OF AET DICHLORIDE FOR NORMAL AND HEPATECTOMIZED FEMALE MICE

	Control	2 Hrs.	1 Day	3 Days	1 Week	2 Weeks
Without conditioning dose	409±52[a] (13)	340±75 (7)	334±24 (6)	381±67 (7)	370±55 (8)	417±29 (8)
After dose of 250 mgm./kgm.	248±51[a] (9)	165±40 (6)	145±35 (6)	240±78 (8)	223±64 (9)	274±63 (10)
Detoxified (mgm./kgm.) in 3 hours	89[a]	74	61	110	103	107
Mortality	0/9[b]	5/11	0/6	0/8	1/10	1/11
% Liver weight (wet)	6.08±.32	2.14±.24[c]	3.70±.38	4.68±.61	5.66±.91	6.43±.81

[a] Mgm./kgm.

[b] Numerator = mortality within three hours after 250 mgm./kgm., denominator = number of animals used.

[c] Includes animals from 1 to 5 hours after hepatectomy.

Four male mice were given 250 mgm./kgm. and kept at 19° C. for three hours. The average accumulated toxic dose was 275 mgm./kgm. instead of 428 mgm./kgm. (Table 2) for male mice kept at 30° C. The importance of environmental temperature is indicated by a comparison between these values.

TABLE 2

ACCUMULATED LETHAL DOSE (MGM./KGM.) AND DETOXIFICATION OF AET DICHLORIDE IN MALE MICE

	Direct	1 Hour After 250 mgm./kgm.	2 Hours After 250 mgm./kgm.	3 Hours After 250 mgm./kgm.
Normal Mice				
Accumulated lethal dose	250[a] + 170±21	220±44	304±49	428±27
Detoxification in stated time	...	50	134	258
Hepatectomized (2 Hours)				
Accumulated lethal dose	250 + 135±69	.....	190±58	235±70
Detoxification	...	.....	55	100

[a] mgm./kgm.

Summary

A method for estimating the in vivo detoxification of AET is presented. A marked sex difference was found with male animals being able to detoxify AET more rapidly. Partial hepatectomy produced about the same decrease in the toxic level for male and female mice. Whereas the decrease in detoxification was not significant for females, there was a marked decrease in detoxification in male mice following partial hepatectomy.

PHARMACOLOGICAL AND TOXICOLOGICAL COMPOUNDS AS PROTECTIVE OR THERAPEUTIC AGENTS AGAINST RADIATION INJURY IN EXPERIMENTAL ANIMALS

V. Effect of Some Protective Agents on Subcutaneous and Intraperitoneal Oxygen Tension of Male Mice

H. D. Landahl

This report concerns: Polarographic measurements of subcutaneous and intraperitoneal oxygen tension following administration of some compounds which protect against radiation lethality.

Immediate or ultimate application of the results: To provide information on the mechanism of action of compounds which protect against the effects of ionizing radiations.

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An attempt has been made to estimate the changes in tissue oxygen tension of mice following injection of p-aminopropiophenone, serotonin, glycononitrile, malononitrile, 2-aminoethylisothiuronium dichloride and mercaptoethylamine hydrochloride. The doses injected correspond to the values used for protection against lethality (1,2). The measurements were mostly made at about twenty minutes following injection since this is approximately the time which elapsed from the time of injection of the drug to the mid-point of the x-irradiation dose.

Enameled platinum wire was threaded through hypodermic needles (24 or 25 gauge) which were then filled with resin while hot. After testing to eliminate the possibility of electrical contact between wire and needle, the needles were baked at about 130° C. overnight.

Two such needles were inserted under the skin of the lower back of a mouse strapped to a thin plate held on a ring stand. Small weights of about 20 grams tied to suture thread were fastened to each foot with a slip knot while the mouse was held in the left hand. The mouse was placed on the plate so that the weight hung over the side thus holding the legs apart. A small strip of aluminum forming an arch in its middle and held tightly on one end was lifted up at the other end while the head of the mouse was put under the strip. The strip was then pressed back to the board with the arch over the neck and held in position with a clip. The front feet were pulled forward over the strip being held by the weights. Two calomel half cells were each attached to a wick and one wet wick was placed over each hind foot and held over the foot by a clip with light pressure.

A modification of the alternating current method (3) was followed to measure the oxygen tension. The cycle time of 15 seconds was divided into four equal periods by means of a commutator. During the first period the positive

current (0.6 volt supply) flowed from the platinum through the tissue to the calomel half cell without passing through the galvonometer. During the second and fourth phases no current flowed. During the third phase the voltage was reversed (0.6 v). During the first part (1/3) of this phase the galvonometer was shorted and during the remainder of the phase the galvonometer was in series. The maximum deflection was sat. It was about 1.33 times the value at the break when the third phase ended. Hence the reading represented the value of the current near the end of the third phase, although the actual value depends on a weighted average of the current from the time the shunt opened and the time of the maximum deflection. The readings were very reproducible and could be read to a half per cent. However, because of numerous factors this precision could not be utilized. When the electrodes were in place in a mouse most successive readings differed by less than 2%. However, slow drifts frequently occurred which occasionally amounted to as much as 40%. There also occurred occasional nearly discontinuous changes of the same magnitude. Movement of the mouse just after a reading often had no effect on the next reading, often changed it by a few per cent and less often changed it by substantial amounts. If the animal moved within a few seconds before the galvonometer reached its maximum, the reading would be substantially increased. These values were not recorded or if recorded were subsequently ignored. If the animal struggled for more than about five seconds, the subsequent readings were generally lower, except perhaps the first reading. If pure oxygen was applied for several minutes when a reading was low, the reading rarely decreased, usually rose slightly and occasionally rose substantially. If the reading was high before administration of the oxygen, the reading usually increased appreciably and occasionally increased very markedly. These generalizations would indicate that the circulation in the immediate vicinity of the electrode varies as is to be expected. For this reason two electrodes were used in parallel.

Since temperature modifies the response of the electrodes and since the drugs result in loss of temperature control, the animals were kept warm while fastened to the plate. During long runs the rectal temperature was observed continually. In most instances a mouse was observed for about six minutes with the results during the first three minutes being discarded. The mouse was injected with the drug (1% of body weight) and placed in a box warm enough to prevent appreciable loss of temperature. Fifteen minutes after injection the polarographic measurements were again made. Rectal temperatures were taken before and after and occasionally during this period.

The results of measurements of oxygen tensions in the subcutaneous regions of the back of the male mouse are shown in Table 1 in microamperes. The values in the fifth column of the table are the ratios of values from treated animals to the control values. In the last column the ratios are corrected for the decrease in the reading occurring upon the addition of the drug to Tyrode's solution in an amount sufficient to make the concentration the same as the dose in the second column. If the drugs were uniformly distributed and the effect on the electrode is the same in vivo as in vitro then the "corrected ratio" of the next to the last column of the table would be appropriate. The corrections are linear interpolations between the effect at 0% oxygen and air at 25° C. assuming that the desired factor is for a value of about 6% oxygen. In the case of p-aminopropiophenone a substantial part of the poisoning effect on the electrode is due to the propylene glycol used as the solvent. Only short term effects are considered here. There are long term electrode poisoning effects which could also cause difficulty.

TABLE 1

INFLUENCE OF DRUGS ON OXYGEN TENSIONS IN SUBCUTANEOUS REGIONS OF BACKS OF MALE MICE

(Values are expressed in microamperes)

Drug	Dose (mgm./kgm.)	Oxygen Tension (μa)		Ratio Control/Drug	Corrected Ratio	Estimated Fractional Decrease in Oxygen Tension
		Control	20 Mins. After Drug			
PAPP in H_2O + propylene glycol	30	0.295	0.255	.83 ± .16	.91	.20
Serotonin	100	0.34	0.335	.98	1.09	0
Glycolnitrile	5.3	0.34	0.31	.90	.93	.15
Malononitrile	5.0	0.31	0.30	.97	.99	0
5% Oxygen	.....	0.28	0.23	.82 ± .05	.82	.38 ± .10
10% Oxygen	.....	0.24	0.22	.91 ± .05	.91	.19 ± .10

Tyrode's solution without glucose at 37° C. and saturated at atmospheric pressure gave an average reading of 0.66 microamperes. In nitrogen the corresponding value was 0.15 giving an average sensitivity of 0.25 μa for an increment of 1% in oxygen subcutaneous tissue fluid concentration. It should be noted that different salts have different effects and there may be substances which poison the electrode. For example, addition of liver homogenates shows a considerable effect. On the other hand, 0.1 M neutral buffer markedly increases the reading especially in the absence of oxygen.

If we assume that there is no effect on the current due to tissue fluid substances, then we obtain from the above considerations a value of about 6% oxygen tension which is a plausible value. However, this may occur due to the compensation of two factors (a) the tendency of small movements due to respiration and heart beat to increase the current and (b) the presence of substances in the tissue fluids that decrease the current.

The values in the last column of the table were obtained assuming that the sensitivity to oxygen in the tissue fluid was the same, after correction of the drug effect, as in Tyrode's solution, provided that the readings were normalized to the average normal current of 0.31 microamperes. Thus the factor in the last column ($\Delta P/P_n$), the relative change in the oxygen pressure due to the drug or treatment, is estimated from the following formula:

$$\frac{\frac{i_n}{I_n}(I_n - I)}{P_n - P} = \frac{\Delta I}{\Delta P}_{\text{coln}} = S \quad \frac{\mu \text{ amp}}{\% \text{ oxygen}}$$

or

$$\frac{\Delta P}{P_n} = \frac{1}{S} \frac{\bar{I}}{P_n} \frac{I_n - I}{I_n} = 2.1 \times (\text{corrected ratio}),$$

where $(I_n - I)/I_n$ is the corrected ratio, S the sensitivity in Tyrode's solution, I_n the normal current, I the current after administration of the drug, P_n and P the oxygen tensions in per cent oxygen before and after injection of the drug. Here S = 0.025, I_n = 0.32 and P_n is taken to be 6% which is a value one obtains on equilibrating air or nitrogen bubbles under the skin of the backs of rats for four hours. It should be emphasized that the calculation represents an attempt to eliminate some of the factors involved and is no better than the assumptions made.

From the standard deviation of the uncorrected ratio for PAPP it can be calculated that the ratio is not quite significantly different from unity. When the correction is made the difference is not significant but it is of interest to calculate the corresponding relative decrease in oxygen tension. The value of 0.20 lies in a fiducial range of 20% from a relative increase of 0.10 to a decrease of 0.5. This points out the difficulty in estimating the changes due to the drug.

The values of the change after administration of 5% or 10% oxygen were more constant since the changes were measured during such a short time. When the animal was quiet and constant readings were being recorded, the reduced oxygen mixture was administered without disturbing the animal for a period of about three minutes. Most of the change was within the first minute. The readings for two minutes just before and for two minutes following three minutes of air were averaged to obtain a reference for the third minute readings with reduced oxygen. In the case of reduced oxygen only three mice were used for each oxygen tension, these being 12 and 17 separate observations for the 5% and the 10% oxygen tension respectively. It can be seen that the drop from 21% to 10% O_2 reduces the subcutaneous tension by about the same factor as does the reduction from 10% to 5% O_2. In this connection it may be pointed out that it requires a substantial reduction in oxygen tension to produce any appreciable protection.

A few observations were made on the intraperitoneal oxygen tension. The control values were not substantially different from the subcutaneous values nor was there any evident change produced after serotonin or glyconitrile had been given.

A few animals which had been given p-aminopropiophenone 15 to 35 minutes previously were given 100% oxygen for three minutes to observe changes in the polarographic readings. The results of four readings were 0, 8, 10 and 33% increases in the readings obtained due to administration of the oxygen. These values are erratic as in the case of normal animals mentioned above. The increases are, on the average, about sufficient to cancel the decrease due to the drug but, in view of the variability in both values, not much can be concluded from this finding.

Although the substances mentioned above produce some inhibiting effect on the platinum electrode, the effect is quickly reversed. However, with a number of sulfhydryl compounds the poisoning effect is not only large but may be very slowly reversed. In order to estimate the oxygen tension following injection, it is necessary to estimate the concentration of the drug near the electrode and then know that the factor to be applied is not modified by other substances which are in the subcutaneous fluid. In the case of the drugs listed above the factor was not too large and, therefore, the assumptions made in using the correction factor are not too restrictive. In the case of the sulfhydryl compounds tested this is unfortunately not true. The estimated values for the oxygen tension are very doubtful. With these qualifications the results of a few observations are given. Using Tyrode's solution at 37° C. the reduction factors for AET (225 mgm./kgm.) and MEA (200 mgm./kgm.) were respectively 0.40 and 0.45 in nitrogen but were 0.31 and 0.33 in aerated solutions. These values are not very sensitive to concentration. A linear interpolation for 6% oxygen gives ratios 0.37 and 0.43. The average oxygen tension current readings ten minutes after injection of AET or MEA were 0.42 and 0.32 respectively relative to the control value. Thus for AET the reduction in vivo is about the same as that in vitro while for MEA the in vivo factor is somewhat smaller than that obtained in vitro. In view of the uncertainties discussed above one cannot conclude that there is any change in oxygen tension following administration of these drugs.

A few observations on the time course of the polarographic readings following intraperitoneal injection of AET showed a rather rapid drop in the first few minutes, there being relatively little change from 15 to 30 minutes but a very slow increase thereafter. The readings made intraperitoneally at twenty minutes were about the same or perhaps higher than the corresponding subcutaneous values. When 70 mg./kgm. were given intravenously over a 30-second interval, the subcutaneous readings were down by the end of the injection, continued to fall rapidly for about a minute, then slowly for several minutes thereafter.

Summary

Polarographic measurements failed to show that serotonin, glyconitrile or malononitrile reduce the oxygen tension of tissues. p-Aminopropiophenone showed a probable decrease. For various reasons it was not possible to eliminate the possibility that PAPP may reduce the tension rather markedly. It was not possible to draw any conclusions regarding the effect of 2-aminoethylisothiuronium dichloride or mercaptoethylamine hydrochloride on the oxygen tension of tissues because the poisoning effect on the electrodes in vivo is comparable to that in vitro.

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PHARMACOLOGICAL AND TOXICOLOGICAL COMPOUNDS AS PROTECTIVE OR THERAPEUTIC AGENTS AGAINST RADIATION INJURY IN EXPERIMENTAL ANIMALS

VI. On the Role of Temperature in Radiosensitivity and Radioprotection in Mice

H. D. Landahl and A. T. Hasegawa

This report concerns: The effect of starvation on temperature control, the effect of body temperature of starved mice on sensitivity to radiation, the effect of radioprotective compounds on temperature regulation and the effect of temperature on the toxicity of some radioprotective compounds.

Immediate or ultimate application of the results: To provide information on the role of environmental and body temperature in radiosensitivity and in the toxic effects of some radiation protecting compounds with a possible resulting increase in the therapeutic index for protection.

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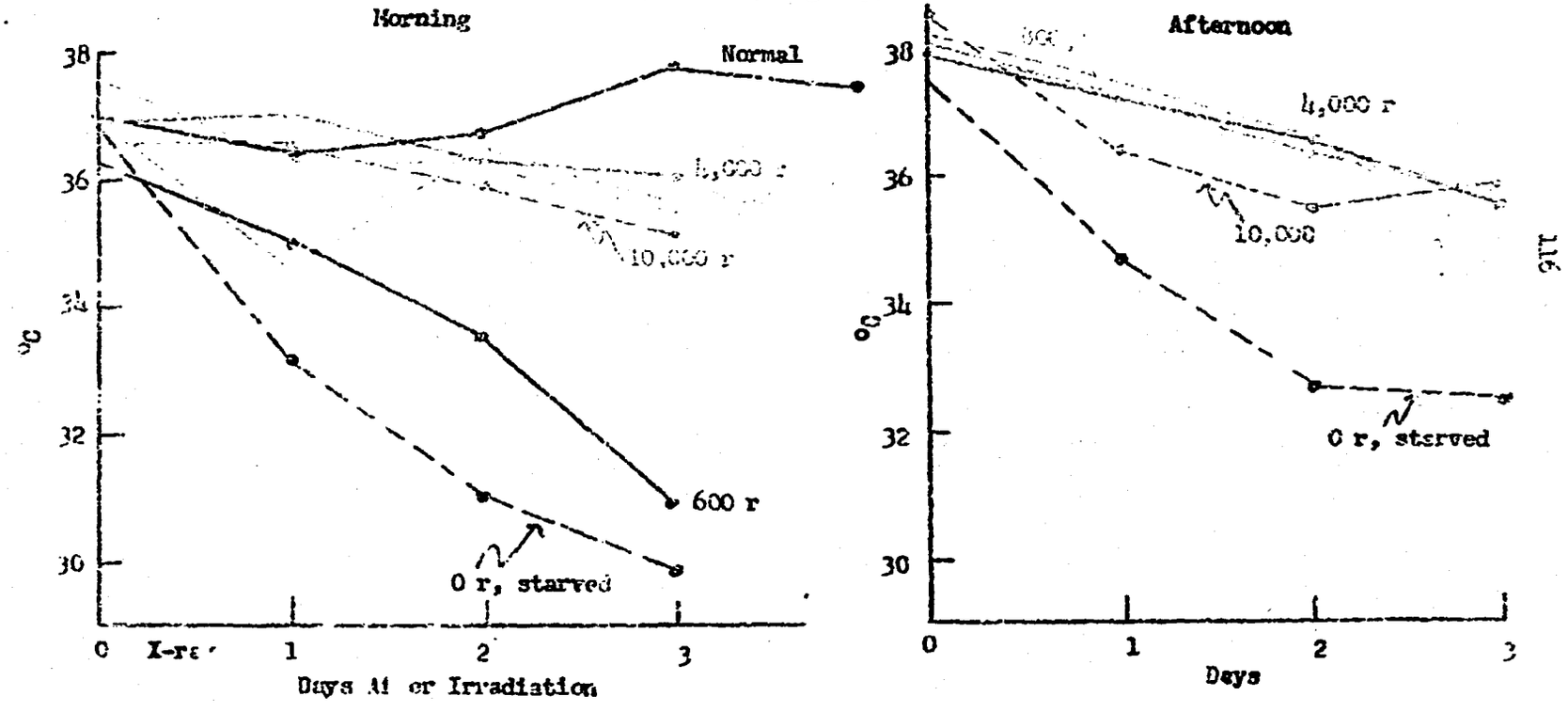
Environmental temperature plays a relatively small role in survival after radiation of normal rats (1). Normal mice, however, maintain their temperature fairly well over a wide range of environmental temperatures. Starved mice, on the other hand, lose the ability to regulate temperature. Furthermore, most radioprotective compounds seriously interfere with temperature control. As a result the body temperature at the time of irradiation can be varied over a wide range. The protective power of a drug can thus be measured for various values of body temperature. The value of the body temperature which is maintained may determine the maximum level of the effective substance as well as the duration of its effect (3). This latter statement implies that, in general, the toxic effect will also depend upon temperature. Since the toxic component of drugs may well be different from the protective component, the time course of the body temperature may affect the toxicity and the protective action differently. Thus it may be possible to maximize the therapeutic ratio for protection by proper choice of the temporal pattern for the body temperature.

Effect of x-irradiation and starvation on rectal temperature of mice kept at room temperature (22° to 26° C.). Female mice were exposed to various levels of x-irradiation and their rectal temperature was followed in the mornings and afternoons. All exposed mice were starved following irradiation. The results of measurements on groups each containing eight mice are shown in Figure 1 where it may be seen that unexposed, starved mice lose temperature control. This is especially marked when measurements are made in the mornings. Care must be exercised to avoid stimulating the mice too much in handling since this can result in a substantial temperature increase. It is also to be noted that the irradiated mice (800 r or greater doses) show only a small loss in temperature.

Figure 1

Rectal Temperature of Female Mice After X-irradiation

(Eight mice per group)



A repetition of the morning measurements was made using four male and four female mice in each group. Two additional groups given 600 r were included. The results were more variable due to the small numbers of animals used but the general results were the same except that the starved control male mice showed less temperature decrease the first day. The readings for the 600 r groups were lower than the values for any other dose with one exception. The average of the results from the four male and four female mice at 600 r have been added to the data of the first experiment and are included in Figure 1.

Effect of x-irradiation and starvation on rectal temperature of mice exposed to heat and cold. Male and female mice, six in each group, were exposed daily to cold after withdrawal of food. The rectal temperatures were recorded every half hour for 2.5 hours. The results are shown in Figure 2. The results for males and females are shown separately and it may be seen that there was little sex difference. The unstarved animals showed a decrease in the first half hour and then only a slight decrease thereafter. In other control groups these trends do not show up. The immediate drop depends upon handling to some extent. If the unstarved mice are handled, their temperatures are slightly increased and this increase rapidly disappears. The effect of handling also shows up in the next days. These mice were handled for other purposes and hence their temperatures were up initially. After one or two days without food the temperature dropped substantially.

Female mice in groups of four were given various doses of x-rays and subsequently exposed to heat or cold. The results of these measurements at the third day after x-ray are shown in Figure 3. All groups behaved essentially the same when exposed to heat except that some of the 10,000 r animals died within an hour after exposure to heat. The reaction to exposure to cold was substantially independent of dose except that again some of the irradiated animals died within three hours after the start of exposure to cold.

It should be emphasized that the temperature changes depend on the extent of handling when the temperatures are taken, the number of mice in each group, the materials from which the cages are made, the presence or absence of drafts and other similar factors. These factors will not be considered here.

Effect of temperature change induced by starvation on survival after x-irradiation. Male mice were starved for two days and then exposed to various levels of x-irradiation while either warmed to normal body temperature or cooled to low body temperatures. One group of mice was kept warm before and during exposure to x-rays. They were exposed in perforated, plastic cartons in a constant temperature box at 30° C. At the end of exposure their temperatures were measured. The mean values for the subgroups exposed to 520 r, 600 r, 700 r and 800 r were 37.6, 37.7, 37.7 and 36.5° C. respectively. Through an error, the box temperature of the 800 r group dropped to 26° C. at the end of the exposure. Mice in a second group were placed in perforated plastic tubes and kept in a constant temperature box at about 5° C. for a half hour and then exposed to x-rays while in the cold box. Rectal temperature was measured at the end of the exposure to x-irradiation. The mean values for the 520 r to 800 r groups were 22.0, 24.5, 26.7 and 27.2° C. respectively. The survival curves are shown in Figures 4 and 5. There were twelve mice in each group except for the 800 r group in which there were eight mice.

Figure 2

Effect of Starvation on Temperature Loss on Exposure to Cold (5° C.)
(Six mice per group)

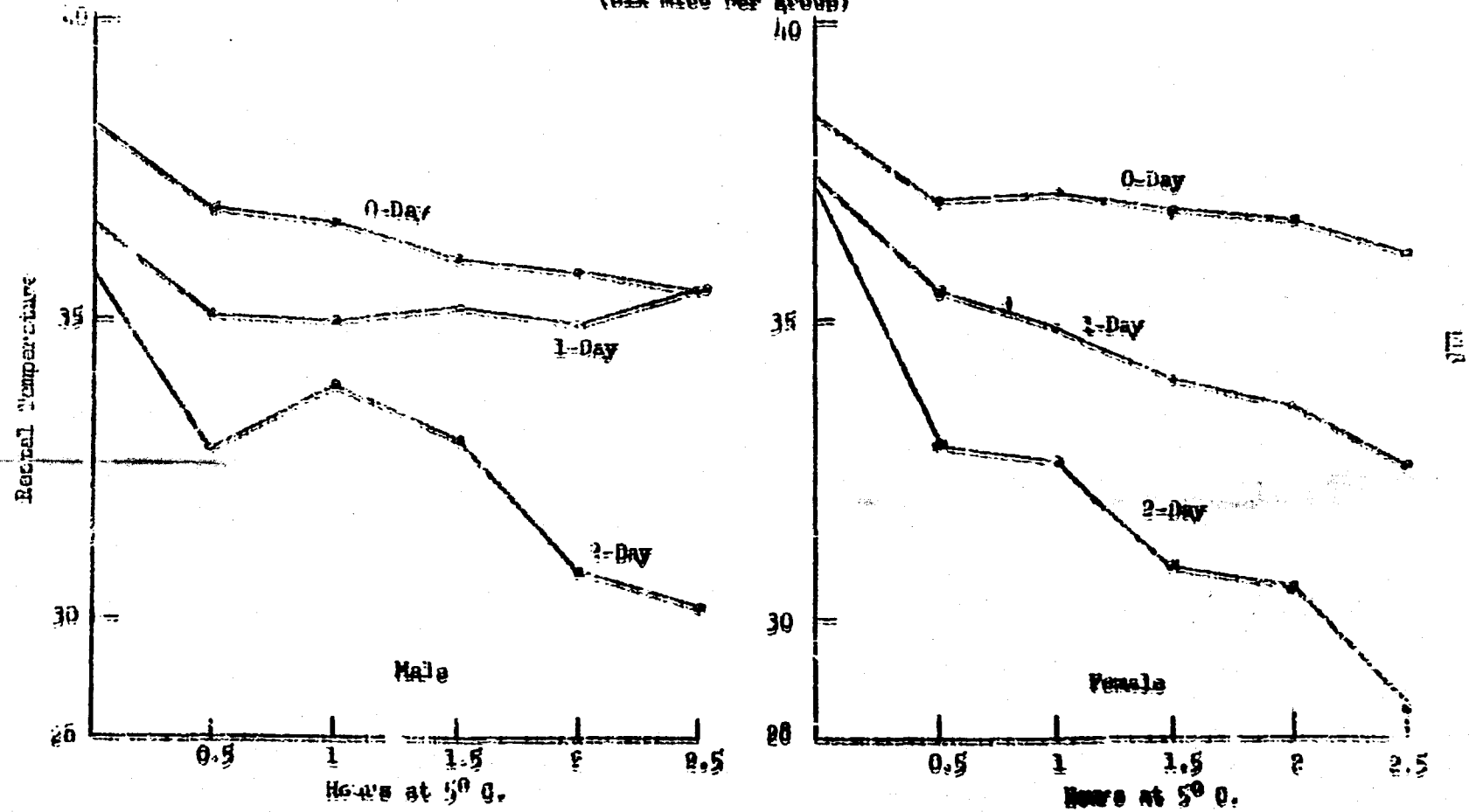


Figure 3

Effect of X-irradiation and Starvation on the Rectal Temperature of Female Mice Exposed to Heat or Cold Three Days After Irradiation

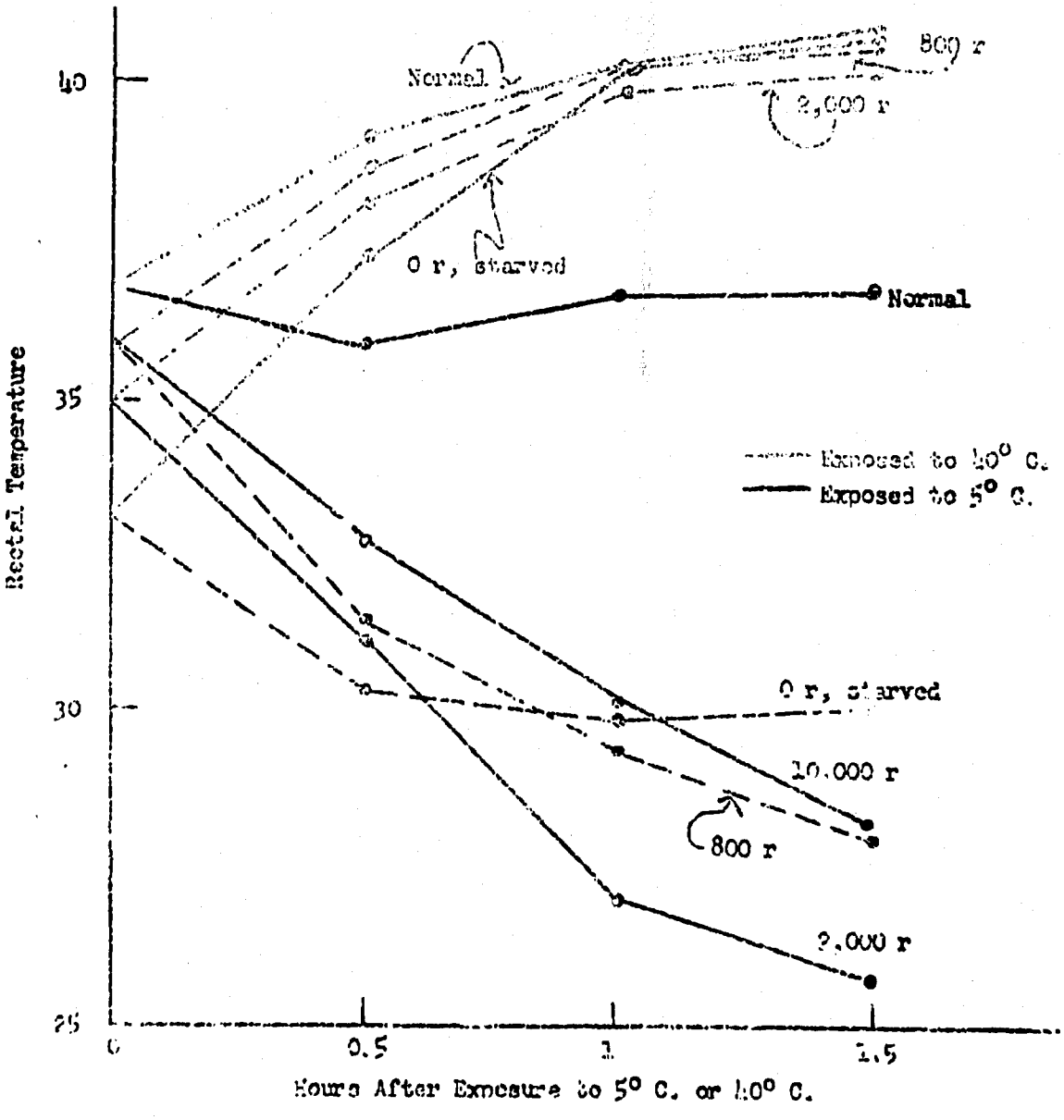


Figure 4

Effect of Rectal Temperature During Exposure to Irradiation on Survival of Starved Male Mice

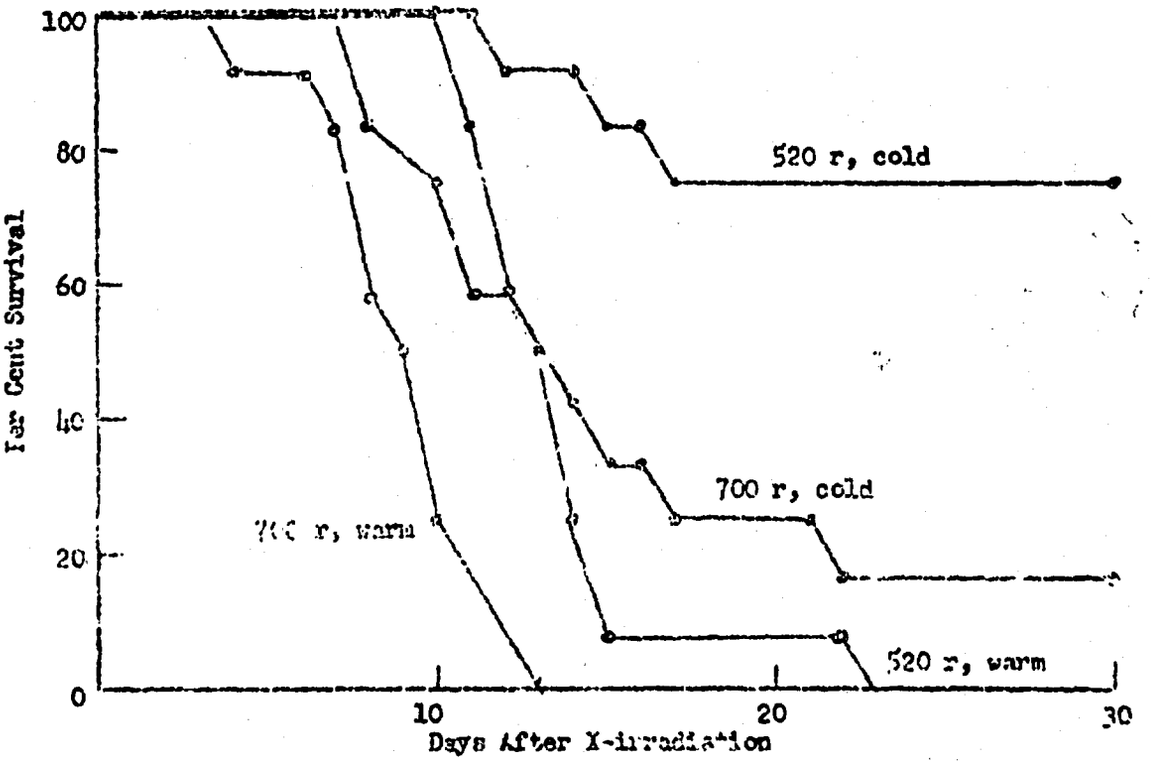
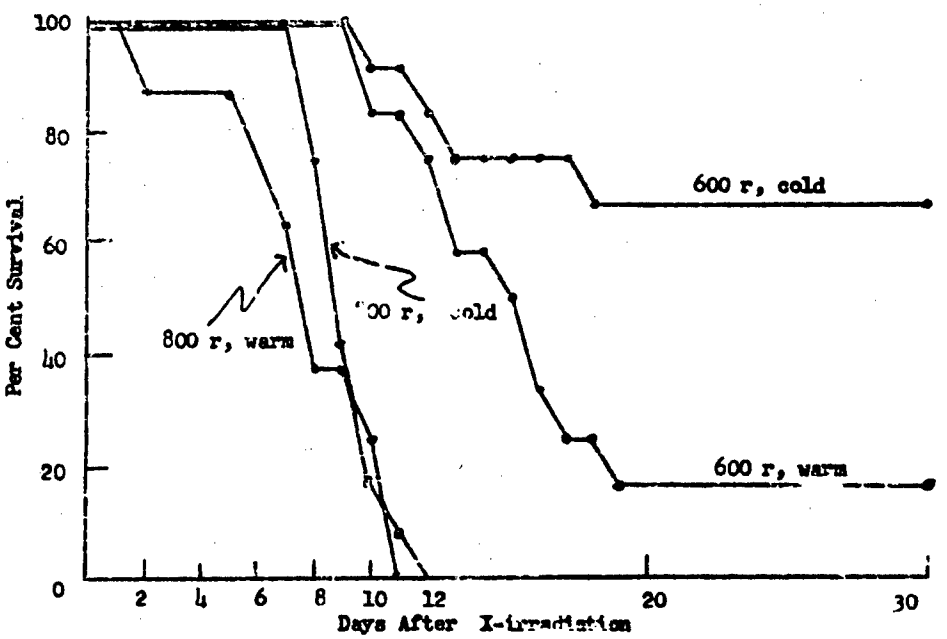


Figure 5

Effect of Rectal Temperature During Exposure to Irradiation on Survival of Starved Male Mice



There was considerable variation in the rectal temperature between individual mice. In large part this was due to the struggling in the plastic tubes. Those mice which struggled longest showed the greatest drop in body temperature. Hence the mice were marked to determine a possible correlation between survival and final rectal temperature. The results were inconclusive. There was only a suggestion that those mice with body temperatures around 25° C. did the best.

A comparison between the above results on starved mice and other data from this laboratory obtained at about the same time indicate that the starved mice which are kept warm are more sensitive to radiation than normal mice. The animals which received 600 r died earlier than normal controls which have an ST_{50} of about nine days. About one-half of the 520 r normal animals would be expected to survive while a few of those receiving 600 r would be expected to survive.

Temperature changes following administration of sublethal doses of radioprotective and related non-protective compounds. Since the body temperatures may play a role in protection and since the extent and duration of the body temperature reflects the in vivo inactivation of an administered drug or its products, it seemed desirable to follow the time course of body temperature following administration of a number of drugs which protect. A temperature of 24° C. was chosen as the environmental temperature for animals following intraperitoneal administration of the various drugs. The results, using three female mice for each drug, are shown in Figures 6, 7 and 8. The numbers after the name of the drug in the figures are the doses in milligrams per kilogram used. These values are those used in the screening program (2,3). The ratios following the dose level are mortality ratios for protected male mice given 800 r. It can be seen from the figures that the compounds which protect all produce a rather marked loss in temperature control when the dose is in the range which shows protection. It may be added that reduced oxygen tension also results in loss of temperature control. Chlorpromazine, which has no appreciable protective value, produces a marked loss in temperature, although more slowly than the others.

Recovery from loss in temperature can be hastened by stimulation. Hence it might have been better to have introduced a low level, periodic stimulation to reduce the time lag between the observed temperature and the intrinsic factors involved in the temperature control. Since a number of drugs were injected over a period of time and the animals were all kept in the same container, the disturbances resulting from measuring rectal temperatures were hardly random. Hence there may have resulted an appreciable amount of systematic error in addition to random error. An idea of the latter variability is indicated from the average standard deviation for the one-hour readings which was 1.5° C. or a standard error of 0.9° C. for the average point at one hour.

Effect of body temperature at time of irradiation on protection. A few preliminary observations have failed to show that the body temperature at the time of radiation has much effect on protection in contrast to the effect in starved animals. Results from sixteen mice given 225 mgm./kgm. of AET twenty minutes before a rapid exposure to x-ray (750 r) showed no difference in survival between those kept at about 25° C. to prevent loss in body temperature and those permitted to drop about 5 degrees before being exposed. Similar

Figure 6

Effect of Various Drugs on Rectal Temperature of Mice at Environmental Temperature of 24° C.

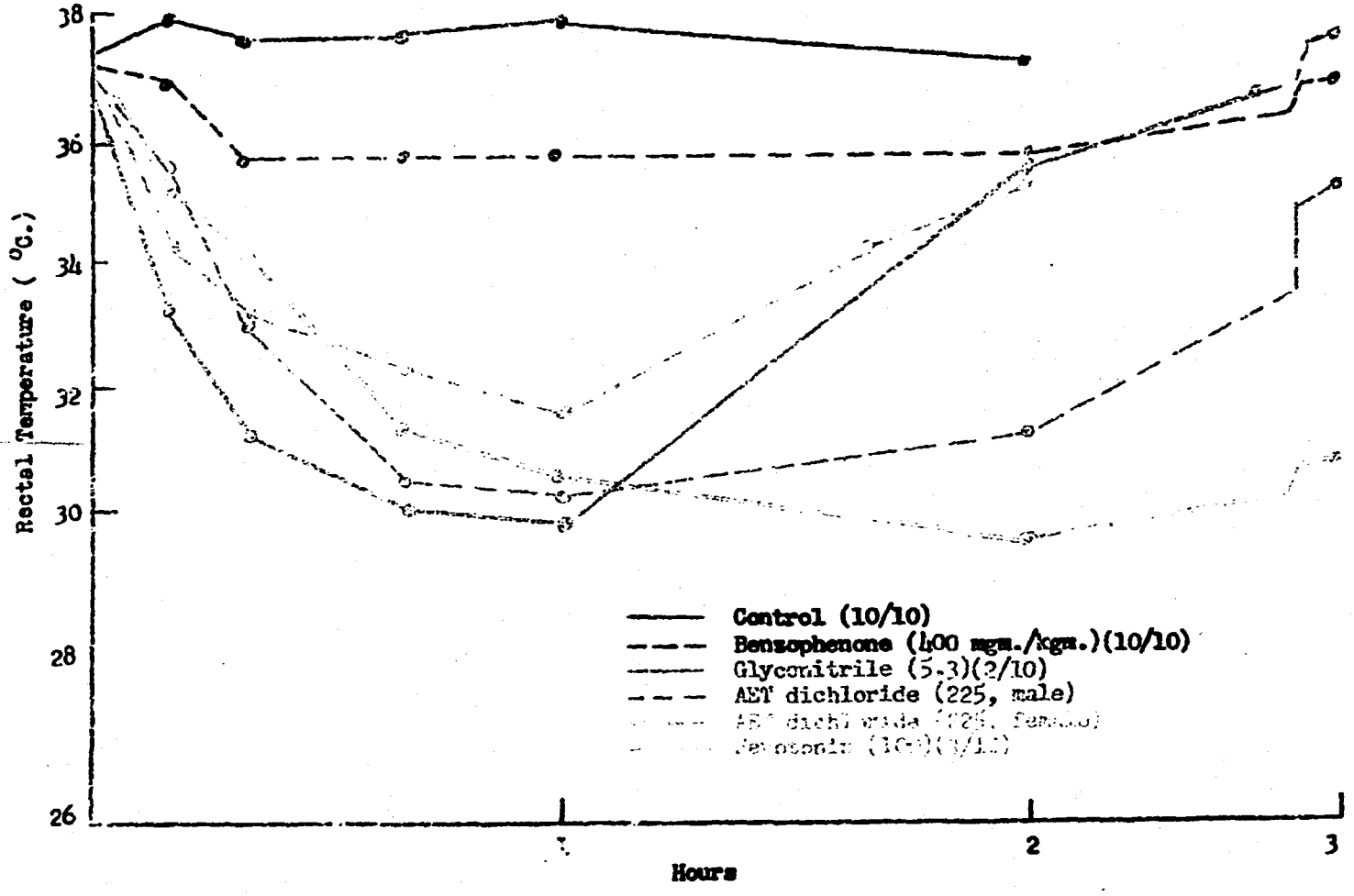


Figure 7

Effect of Various Drugs on Rectal Temperature of Mice at Environmental Temperature of 24° C.

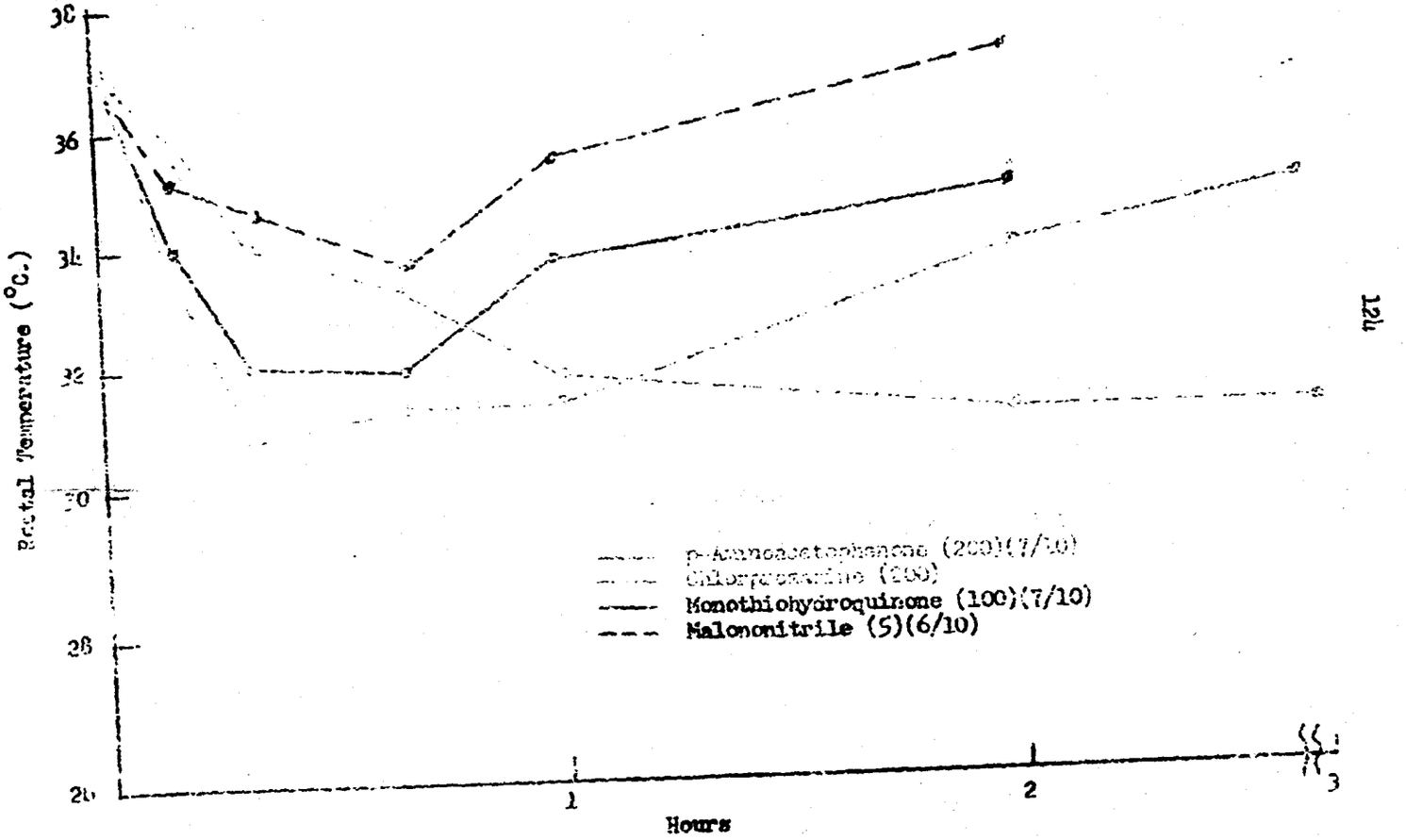
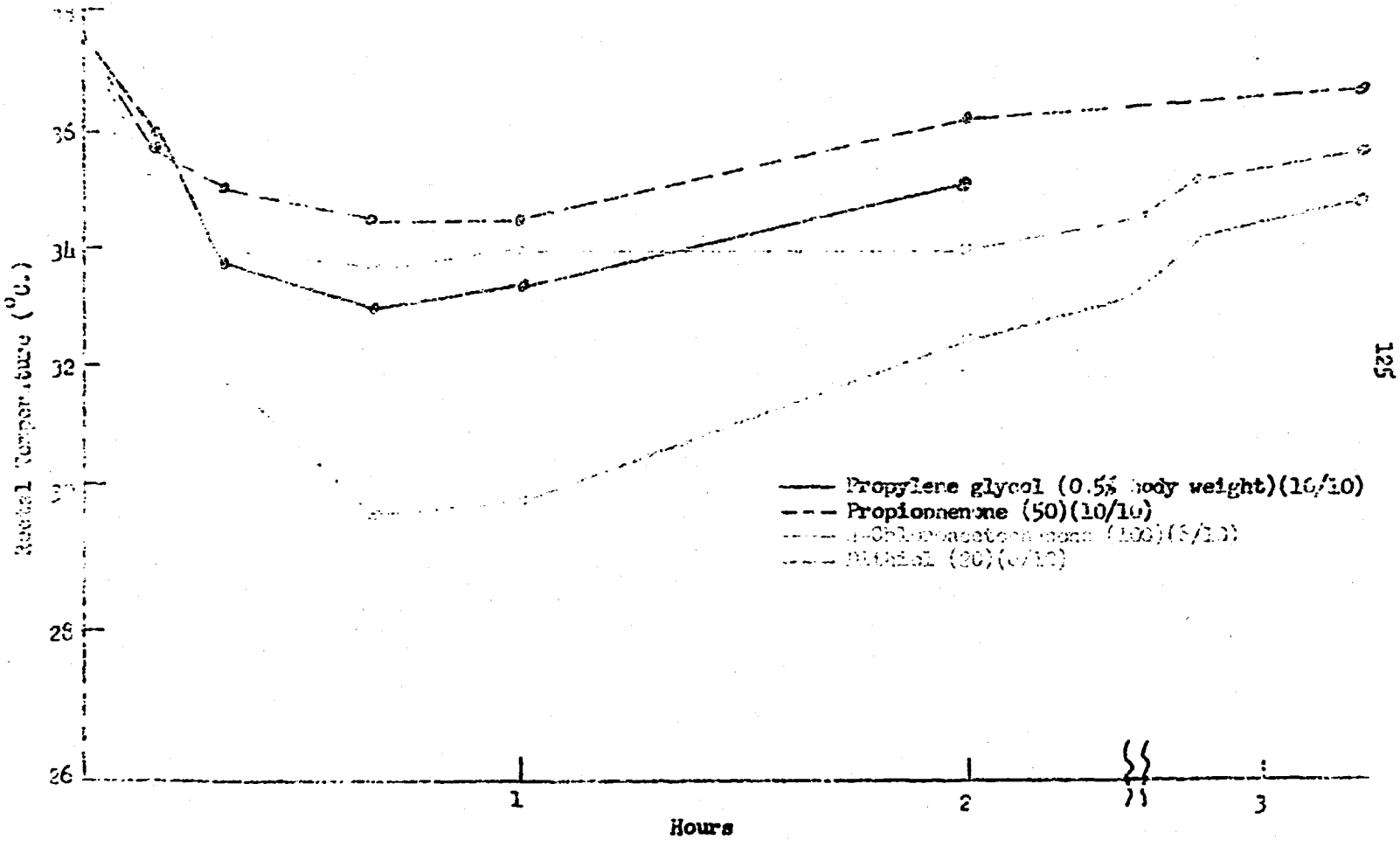


Figure 8

Effect of Various Drugs on Rectal Temperature of Mice at Environmental Temperature of 24° C.



treatment with eight mice per group using propylene glycol (1% body weight) failed to show any difference between those kept near 37° C. and those chilled so that their temperature dropped about 6° C. In the case of male mice pretreated (20 minutes) with PAPP and then given 840 r the thirty-day survival for the warm (36° C.) and the cold (about 31° C.) were both 6/12. In the case of females the corresponding survivals were 2/12 and 6/12 suggesting a slight beneficial effect of cold in this case. Another experiment using 700 r gave 6/8 and 8/8 surviving for those at 37° C. and 31° C. respectively. Note that elsewhere in this report results with serotonin fail to show that decrease in body temperature at the time of radiation increases protection. It should be emphasized that these preliminary results are inadequate to draw any conclusions.

Effect of environmental temperature on toxicity of AET and MEA. The therapeutic ratio can be increased either by increasing the protective power of the drug or by decreasing the toxicity. The following observations may contribute towards the latter goal. Some preliminary experiments on the effect of environmental temperature on the toxicity of AET and MEA are given in Table 1. The animals given AET at 30° C. were kept in a constant temperature box for three hours or longer, then carefully transferred to cages which were kept fairly close to 30° C. for a week. Most deaths occurred within the three-hour period, there being almost no deaths after the first day. The animals given AET at 19° C. were kept at this temperature for 48 hours in metal cages, with one or two animals to the cage. They were then transferred to the animal quarters (20 to 26° C.). At the highest dose most deaths occurred on the first day while at the lowest dose most deaths occurred on the third day. In this latter case the animals lost temperature and those failing to recover temperature control by the second day were likely to die soon after being placed in groups in a slightly warmer place. The data indicate that the LD_{50} at 30° C. is about 420 mgm./kgm. while at 19° C. the value is about 260 mgm./kgm. In the former case the animals c.. not be handled to any extent or they will convulse. There are some indications that by keeping them cool at first they can be handled with less risk.

In the case of the data on MEA the animals were treated the same as those given AET except that they were kept at 30° C. in the constant temperature box for a minimum of twelve hours. The animals given MEA at 22° C were kept at this temperature for about four hours ther left in a room of... temperature ranging from 20 to 25° C. The data in Table 1 indicate that the LD_{50} at 30° C. is about 380 mgm./kgm. while at 22° C. it is about 530 mgm./kgm. The value at 22 degrees is not necessarily the minimum value, known presumably at substantially lower temperatures the toxicity is increased.

The above results indicate the important role that environmental temperature can play in the toxicity of these drugs. Note that both produce pronounced loss of temperature control which may accentuate the effect of environmental temperature.

Summary

1. Starved mice rapidly lose the ability to control body temperature. Exposure to more than 600 roentgens tends to counteract this effect.

TABLE 1

EFFECT OF ENVIRONMENTAL TEMPERATURES ON TOXICITY OF MEA AND AET

MEA (mgm./kgm.)		300	350	400	500	600	...	...	...	...	...	...
Mortality ratio	At 22°C.	0/18	....	0/6	0/8	11/11	...	...	...	...	...	...
	At 30°C.	1/10	2/10	7/10	...	4/4	...	...	...	...	...	...
AET (mgm./kgm.)		225	250	275	300	325	350	375	400	425	450	475
Mortality ratio	At 19°C.	1/6	3/10	7/10	...	....	...	...	...	...	...	...
	At 30°C.	...	....	0/6	0/6	5/18	2/9	2/9	3/9	5/9	6/9	5/6

2. Starved animals kept at normal body temperature are more sensitive to x-rays than are those which are allowed to lose body temperature. This effect is seen at exposures from 520 to 800 r.

3. Animals given protective doses of a number of protecting drugs all showed loss of temperature control. Most of the drugs indicated a start toward recovery of lost temperature at two hours.

4. Environmental temperature has a pronounced effect on the toxicity of AET and MEA. Changing the environmental temperature from 19 to 30° C. increases the LD_{50} for AET by about 60%. Changing the environmental temperature from 30 to 22° C. increases the LD_{50} for MEA by about 45%.

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THE INFLUENCE OF EXPOSURE TO LOW LEVELS OF GAMMA AND FAST NEUTRON IRRADIATION ON THE LIFE SPAN OF MICE

I. Studies on the Use of Daily Radiation to Death as a Means of Evaluating the Effects of Fractionation, Protraction and Radioprotective Agents on the Life Span of Irradiated Mice

J. Noble, J. Doull, V. Plzak, M. Root and J. Cowan

This report concerns: Preliminary studies to determine the median survival time of mice given radiation exposures under varying conditions and subsequently exposed to daily gamma irradiation at a dose rate of 225 rep/week.

Immediate or ultimate application of the results: It is anticipated that the techniques described in this report will permit a more rapid evaluation of the effects of fractionation, protraction and radioprotective agents on the residual injury following radiation exposure. Since fewer animals and considerably less time are required in this procedure for obtaining life span data, the evaluation of the various parameters influencing chronic irradiation studies may be facilitated.

* * * * * * * * *

Previous studies in this laboratory (1,2) have demonstrated that daily radiation to death at the rate of 40 r/day (whole body x-irradiation) beginning three to four weeks after a primary or test exposure can be used to obtain comparative data on the life span shortening produced by the primary radiation exposure. This procedure is similar to the split-dose technique except that the second dose of radiation is replaced by daily radiation to death. The residual injury is estimated on the basis of the shortening of the median survival time rather than by determining the shift in the LD_{50} of the irradiated animals. Using this technique, it was found that x-ray doses of 100 r to 400 r produced a decrease in the ST_{50} of 15% to 37% and that the effect was related to the test dose administered (1). Further studies demonstrated that the protraction of the test dose through twenty days reduced the effect on the ST_{50} by nearly 35% and that the effect of protraction was independent of the size of the test dose within the range employed (2). Since these studies indicate that life span shortening may be investigated by this procedure and that modification of the test dose can be detected and quantitated, it was of interest to investigate the effects of some of the radioprotective agents on life span shortening. For these studies mice were exposed to radiation doses which would be expected to produce minimal acute radiation mortality and the ST_{50} of the treated animals was compared with that of untreated control groups. Since the cobalt 60 facility of this laboratory is more suitable for studies of this type, all of these studies have been conducted using gamma rather than x-irradiation. In addition, studies are in progress to compare the effect of 10-day, 20-day, 40-day and 60-day protraction of the radiation dose on the ST_{50} of gamma

irradiated mice. Since considereble information on the effects of dose protraction has been obtained in this laboratory and in others, the validity of the present technique can be verified from these studies. This would be facilitated, however, if information on the LD_{50} for protracted radiation exposures under our experimental conditions were available. The present report contains the results of several preliminary studies to obtain this information.

Materials and Methods. Adult male and female CF_1 mice were employed for these studies. The animals were all approximately sixty days of age at the time of the first radiation exposure (primary dose). Both the control and experimental groups were selected from single shipments of mice in order to minimize variation in age, weight and general physical condition. The animals were housed in stainless steel mesh bottom cages and provided with food (Rockland Mouse Pellets) and water ad libitum throughout the study. Each group contained originally sixteen animals divided into two groups each of which was housed separately.

The radiation exposures were administered in the cobalt irradiation facility of this laboratory (3) and the distance of the mice from the 10 curie Co^{60} source was adjusted so that the daily irradiation was given at the rate of 5.36 rep/minute. Since the mice were exposed for seven minutes each day, six days a week, the total weekly irradiation dosage was 225 rep of whole body gamma irradiation. The mice were irradiated in individual plastic tubes which were placed in a circle having the source as its center. Dosimetry measurements were made at each animal exposure position using a 25 r Victoreen Ionization Thimble provided with a 0.5 mm. lucite cover and it was found that there was no significant variation in the dosage rate received at any of the irradiation positions. The duration of each daily exposure was controlled by means of an interval timer which actuated the elevating mechanism for the source and insured the accuracy of the radiation exposure period to less than one second. The environmental temperature of the irradiation facility was maintained between 75° F. and 80° F. and the same temperature was maintained within the animal rooms where the mice were kept between radiation exposures.

To determine the effect of pretreating the animals with radioprotective agents, groups of sixteen male mice were given the following compounds ten to fifteen minutes prior to the administration of the test radiation dose: 2-aminoethylisothiuronium (225 mgm./kgm. of dichloride salt), p-aminopropiophenone (30 mgm./kgm.), serotonin (100 mgm./kgm. of the creatinine sulfate complex) and hydroxyacetonitrile (6.0 mgm./kgm.). The mice were then exposed to the test radiation doses of 100 rep through 1,100 rep of whole body gamma irradiation. Control mice (injected with the vehicle only) were simultaneously exposed to radiation doses of 100 rep to 600 rep and the treated and control groups of mice were then permitted to recover for four weeks following which time both groups were exposed daily to 37.8 rep of gamma radiation six days a week until death. Mortality observations were made daily and probit mortality plots were constructed to determine the ST_{50} for each of the control and treated groups.

To investigate the effects of protraction on the residual injury and recovery rate, groups of twelve female mice were given radiation exposures of 107 rep, 215 rep, 322 rep, 429 rep and 536 rep administered (1) as a single radiation exposure, (2) over a period of ten days, and (3) over periods of

forty and (4) eighty days. The mice in each of these four groups were allowed to recover for four weeks following their last radiation exposure and were then given daily radiation to death in the same manner as described above. An additional control group of mice which received no last dose of radiation was also included in these studies. The median survival time for each of these groups was then computed using probit mortality plots. Statistical analysis of the survival data was then carried out according to methods previously described (1,2).

Experimental

Use of the daily irradiation to death technique to evaluate the residual injury in mice exposed to various doses of whole body gamma irradiation with and without the prior administration of various radioprotective agents. For these studies groups of mice (each of which contained sixteen animals) were given protective doses of 2-aminoethylisothiuronium (AET), p-aminopropiophenone (PAPP), hydroxyacetonitrile (HAN) and serotonin (5-HT) immediately prior to the administration of single whole-body gamma exposures of 100 r to 1000 r and after a delay (recovery period) of four weeks were irradiated daily until death. A similar group of sixteen mice were injected with the suspending vehicle for each of the protective agents and irradiated simultaneously with the treated mice. The control group of mice for these studies received no primary dose of irradiation but were started in the irradiation to death cycle at the same time as the treated and irradiated groups. Unfortunately it has been necessary to repeat the irradiation to death in the control group (0 r) due to the accidental loss of mice from several of these groups and hence the results of the repeat experiment are not yet complete and the survival data on these animals is not included in the graphs. The results of the studies in the remaining group are shown in Figures 1 through 5.

In Figure 1 is shown the effect of increasing doses of gamma irradiation on the survival of mice exposed to daily gamma irradiation to death. Half of the mice exposed to 500 r and 700 r of gamma irradiation died within the 4-week recovery period. With the lower doses of primary gamma irradiation there was a shortening of the median survival time which was related to the magnitude of the primary radiation dose. A probability plot of the number surviving against the days of successive daily irradiation provided an ST_{50} of 14.3 weeks for the mice given 100 r in the primary dose, 12.9 weeks for the 200 r group, 12.5 weeks for the 300 r group and 11.0 weeks for the 400 r group.

In Figure 2 is shown the survival data obtained in mice which were given 6.0 mgm./kgm. of hydroxyacetonitrile prior to the administration of the primary dose of gamma irradiation. The ability of this compound to protect against the acute lethality of whole body x-irradiation is evident in that the animals exposed to 500 r and 700 r exhibited a mortality of 12% and 19% respectively whereas the control mice given these doses of gamma irradiation experienced a mortality of nearly 50%. The range of radiation dosage levels employed for these studies is not sufficiently large to permit the calculation of dose reduction factors based on the 30-day mortality but it might be estimated from the available data that the HAN administration reduced the radiation dosage by about half in terms of the acute lethality. Subsequent exposure of these animals to daily gamma irradiation to death, however, does not provide any evidence for

Figure 1

Mortality in Mice Exposed to Varying Doses of Whole Body Gamma Irradiation and Subsequently Given Daily Irradiation to Death

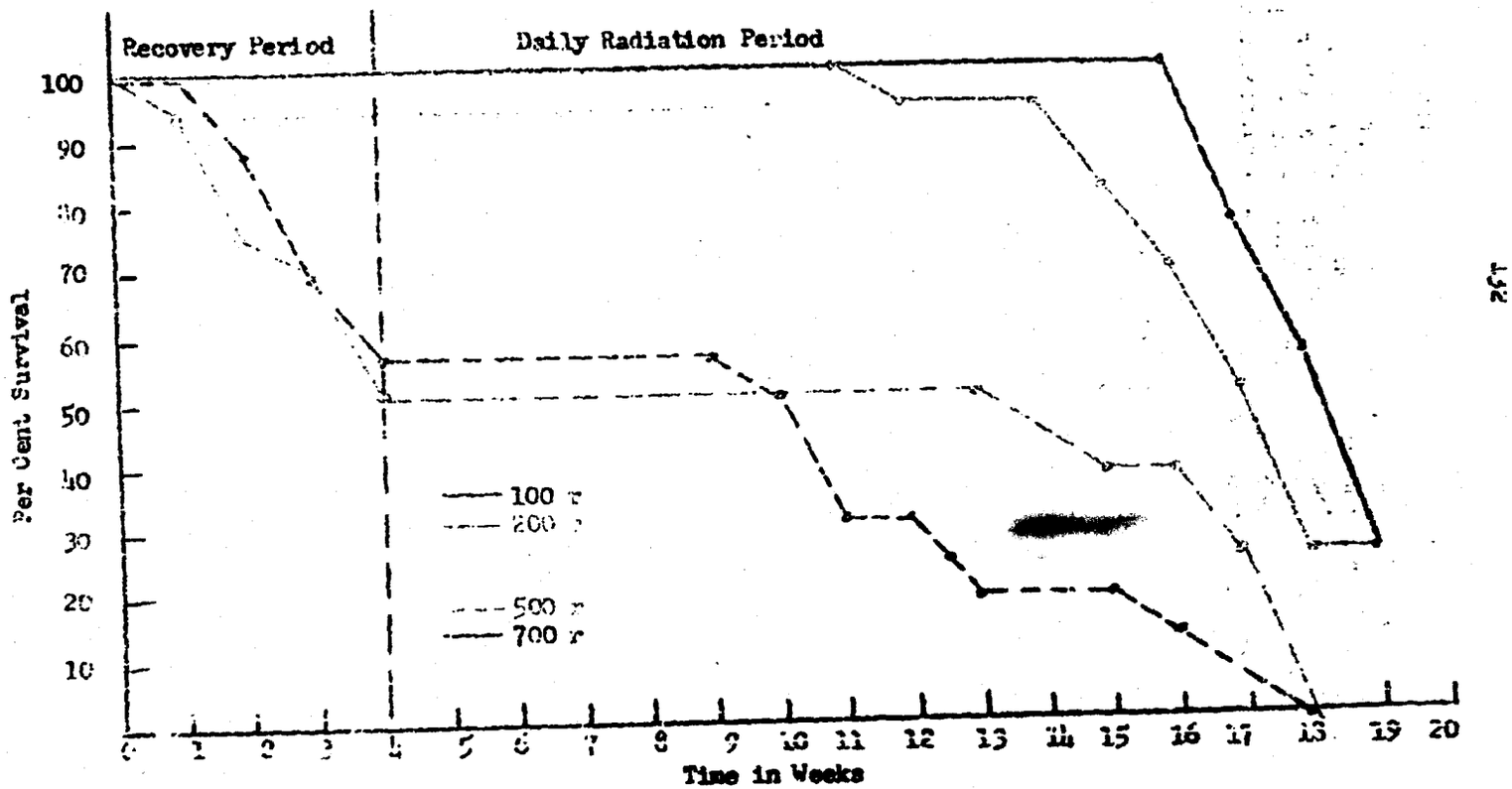
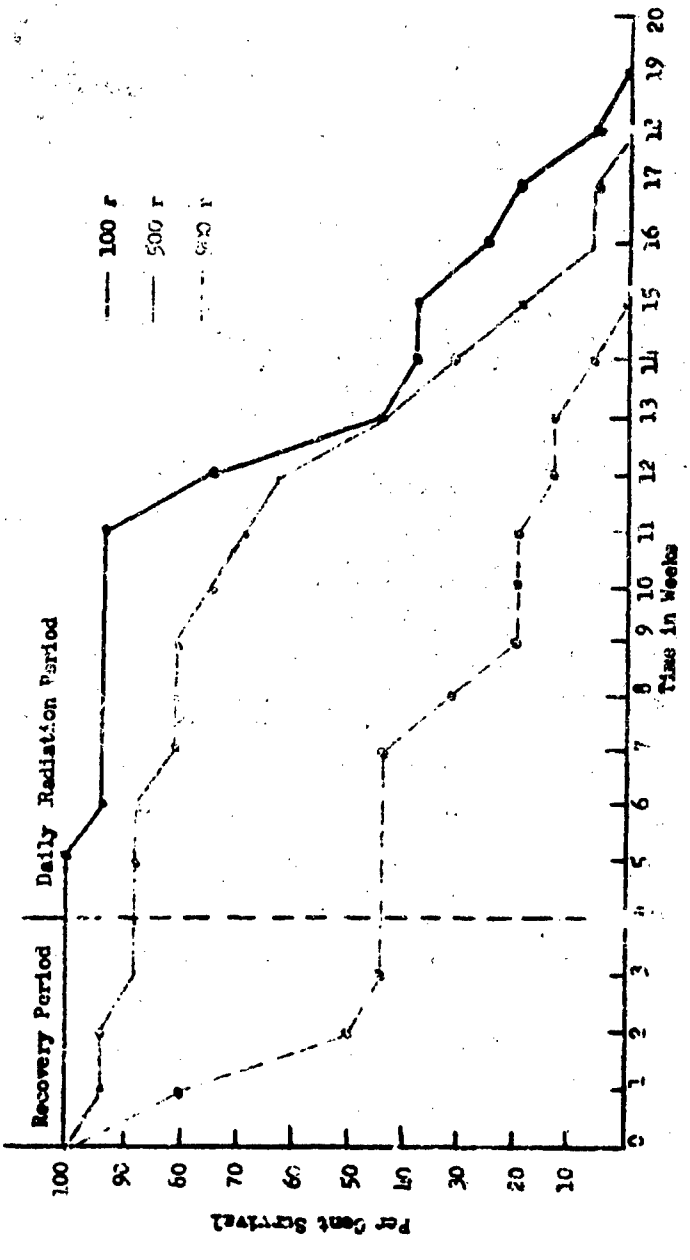


Figure 2

Effect of Hydroxyacetonitrile on the Survival Time of Mice Exposed to Varying Doses of Whole Body Gamma Irradiation and Subsequently Given Daily Irradiation to Death



protective effects against life span shortening. Probit mortality plots of the data included in this graph indicated that the LT_{50} for the mice given 100 r of gamma irradiation was approximately 9.8 weeks, that of the mice exposed to 300 r was 9.0 weeks, and that the ST_{50} of the mice exposed to 500 r and 700 r of gamma irradiation was 8.5 and 6.2 weeks respectively. Thus the median survival time of mice treated with hydroxyacetonitrile prior to the administration of various primary radiation exposures is shortened in comparison to that of animals given the same radiation doses without the protective treatment. These studies would suggest that although HAN is effective in reducing the 30-day mortality of whole body radiation exposure, it does not prevent the chronic irradiation effects of radiation exposure (at least in so far as life span shortening is indicative of such effects).

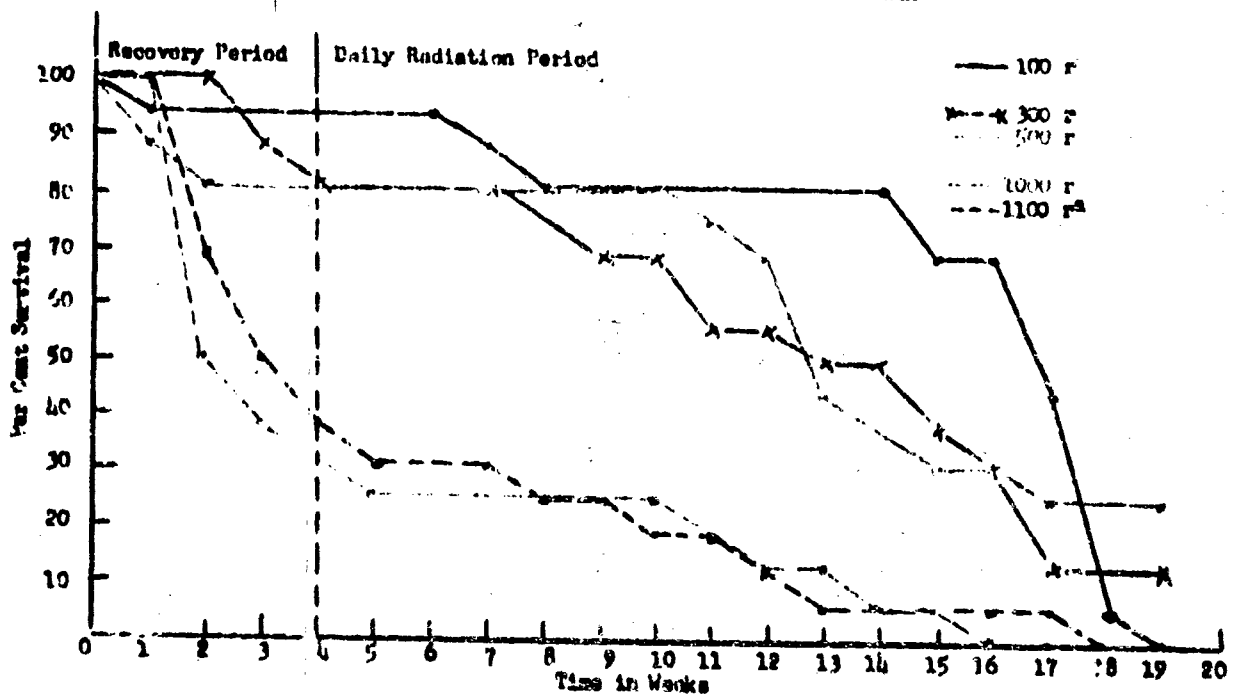
In Figure 3 is seen the results of similar experiments carried out to evaluate the ability of p-aminopropiophenone to provide protection against the shortening of the life span by whole body radiation. The ability of PAPP to partially protect against the acute lethality of whole body irradiation is evident when the mortality of the PAPP-treated animals is compared at thirty days with that of the controls given the same radiation doses. However, the ST_{50} of the PAPP-treated mice subjected to the daily irradiation to death technique was not as long as that of the control animals given comparable doses of radiation within the protective treatment. Thus, the ST_{50} of the mice exposed to a primary dose of 100 r after the PAPP treatment was 12.4 weeks and the PAPP-treated mice which received a primary dose of 200 r exhibited a median survival time of 10.0 weeks. ST_{50} values of 9.0 and 9.2 weeks were obtained in the mice which were treated with PAPP and then given primary radiation doses of 300 r and 500 r. It is evident from these results that PAPP is capable of protecting irradiated mice against some of the acute lethal effects of radiation exposure but that it does not prevent the life span shortening associated with radiation exposure.

Radiation dosage levels of 100 r, 200 r, 500 r, 700 r, 900 r and 1000 r were used to investigate the ability of AET to prevent life span shortening in irradiated mice. The results of these studies are shown in Figure 4. The marked radioprotective activity of this compound against the acute lethal effects of radiation exposure is evident when the mortality of the AET-treated mice is compared with that of the control irradiated groups at thirty days after the irradiation exposure. From the results presented in Figures 1 and 4 it can be estimated that the AET administration reduced the effective radiation dose by about 50%. Although the survival data are still incomplete, it appears that the AET administration also caused some prolongation of the median survival time when the animals were given daily irradiation to death. In the animals which were treated with AET prior to a primary radiation exposure of 100 r, the ST_{50} was 15.2 days. Dosage levels of 200 r, 500 r and 700 r of gamma irradiation resulted in ST_{50} values of 14.9 weeks, 13.0 weeks and 12.3 weeks in the AET-treated animals. The effective dose reduction factor present under these experimental conditions cannot be determined at present since insufficient mortality data has been accumulated.

All of the groups of mice which were treated with serotonin prior to the primary radiation exposure exhibited early mortality due to the chemical toxicity of the drug and it will be necessary to repeat these studies using a lower dosage level of serotonin. It can be seen, however, from the results

Figure 3

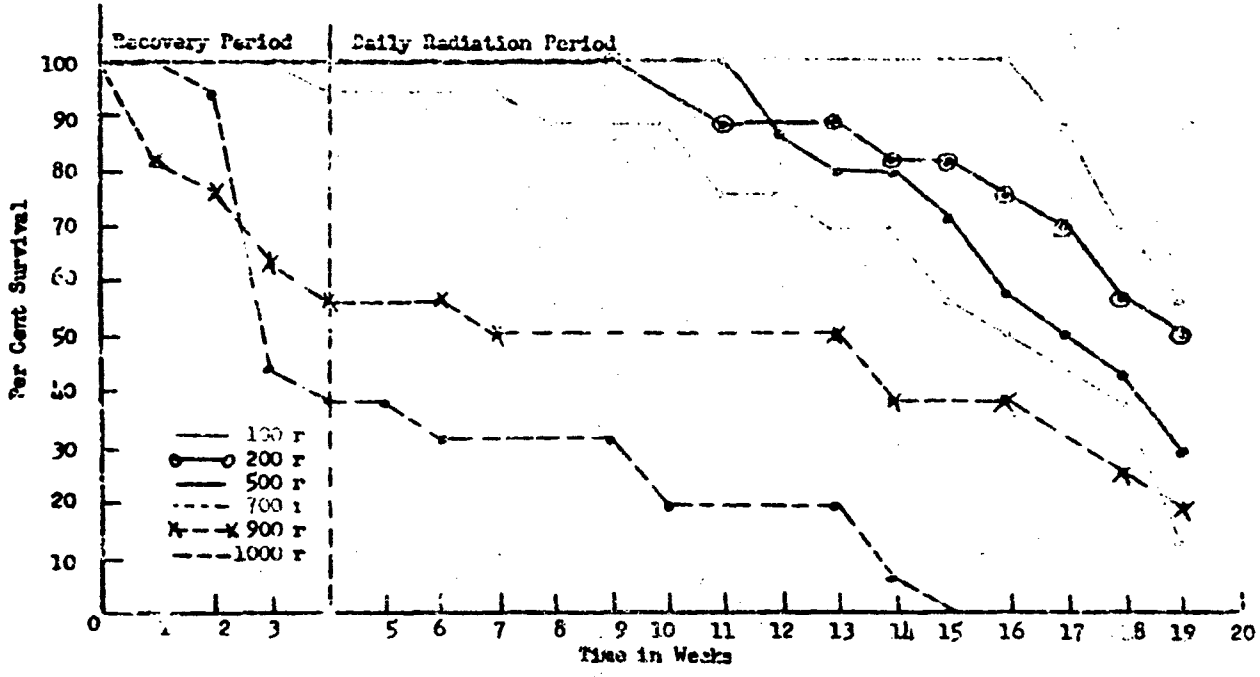
Effect of p-Aminopropiophenone on the Survival Time of Mice Exposed to Varying Dosage Levels of Whole-body Gamma Irradiation and Subsequently Given Gamma Irradiation to Death



*Given in two doses of 550 rep twenty-four hours apart.

Figure 4

Effect of 2-Aminoethylisothiuronium on the Survival Time of Mice Exposed to Varying Doses of Whole-body Gamma Irradiation and Subsequently Given Daily Irradiation to Death



presented in Figure 5 that the ST_{50} of the serotonin-treated mice given a primary radiation exposure of 100 r was about thirteen weeks and that the median survival time of the mice exposed to the 300 r primary radiation dose was about twelve weeks. Thus it would appear on the basis of these studies that serotonin administration does not prevent the life span shortening following radiation exposure in mice.

The results of the studies included in this report are preliminary and additional studies are in progress in which a wider range of dosage levels are being tested. Analysis of the life span shortening data from these studies and the additional studies in progress must await the accumulation of mortality data from the 0 r control groups. It is possible, however, to obtain some indication of the effects of these protective agents by plotting the median survival time of the treated and control groups against the primary irradiation dosage level. In Figure 6 is shown the results obtained with the protective agents PAPP, HAN and AET. It can be seen that the dose response curves are approximately linear. It is also evident that the treatment with AET prolongs the life span of the irradiated mice at all of the dosage levels employed (primary irradiation) while treatment with either PAPP or HAN shorten the median life span of the irradiated animals.

Effect of protraction of the primary radiation exposure on the median survival time of animals given daily radiation to death. Three series of animals were included in these studies. Each series contained five groups of twelve female mice which were given total primary radiation exposures of 107 rep, 214 rep, 322 rep, 429 rep and 536 rep of whole body gamma irradiation. Series A received these doses in a period of ten days, series B received the same doses over a period of forty days, and series C were given these radiation exposures in a period of eighty days. Four weeks after the last radiation exposure all of the animals were started on the daily radiation to death program at a dose rate of 37.5 rep/day for six days a week giving a total weekly radiation dose of 225 rep. At the present time these animals have accumulated about ten weeks of daily radiation and mortality is beginning to appear in the groups which were given the largest primary radiation exposures. Since there is little difference currently between the 10- and 40-day protraction series, it is apparent that any evaluation of the protraction effects are premature. The statistical analysis of the mortality data from these studies will appear in subsequent reports from this laboratory.

In addition to investigating the effects of protraction by means of the daily radiation to death procedure, studies have also been initiated to determine the LD_{50} for protracted gamma radiation exposures in the hope that this information can be used to evaluate the validity of the radiation to death technique as a means for measuring residual injury in chronically irradiated animals. For these studies groups of male and female mice, each of which consisted of 16 or 32 animals were exposed to daily doses of 90 rep, 100 rep, 120 rep, 130 rep, 140 rep, 150 rep, 160 rep and 180 rep of gamma irradiation for a period of ten days and the mortality recorded during the subsequent 30-day period. The results of these studies are shown in Figure 7 in which the per cent mortality (in probits) at thirty days is plotted against the log of the total radiation dose administered. The LD_{50} under these experimental conditions was found to be 1350 rep. A second series of five groups of mice has been given daily radiation doses of 50 rep to 100 rep for a period of

Figure 5

Effect of Serotonin Administration on the Survival Time of Mice Exposed to Varying Doses of Whole-body Gamma Irradiation and Subsequently Given Daily Irradiation to Death

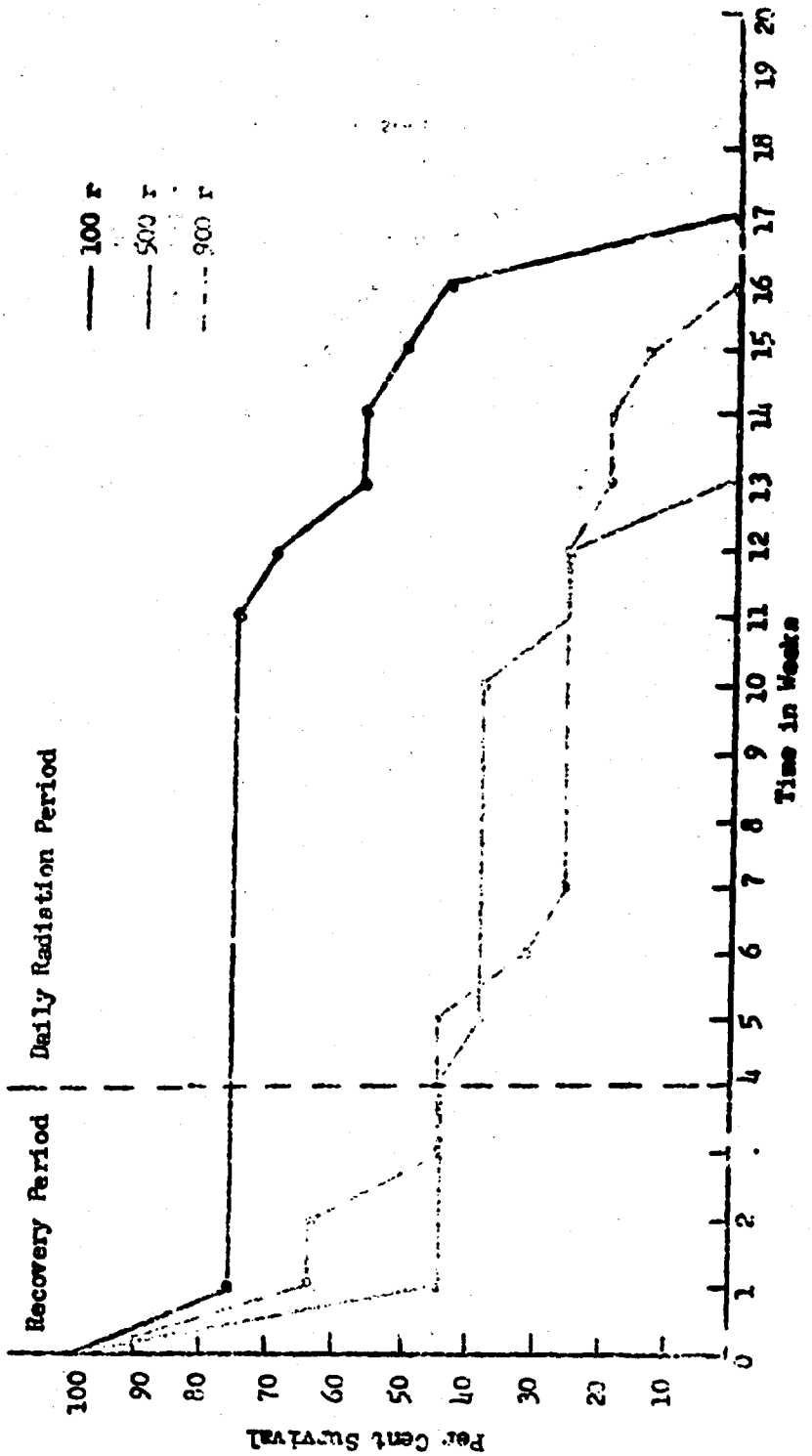


Figure 6

Effect of Various Radioprotective Agents on the Median Survival Time of Mice Exposed to Varying Doses of Whole-body Gamma Irradiation and Subsequently Given Daily Irradiation to Death

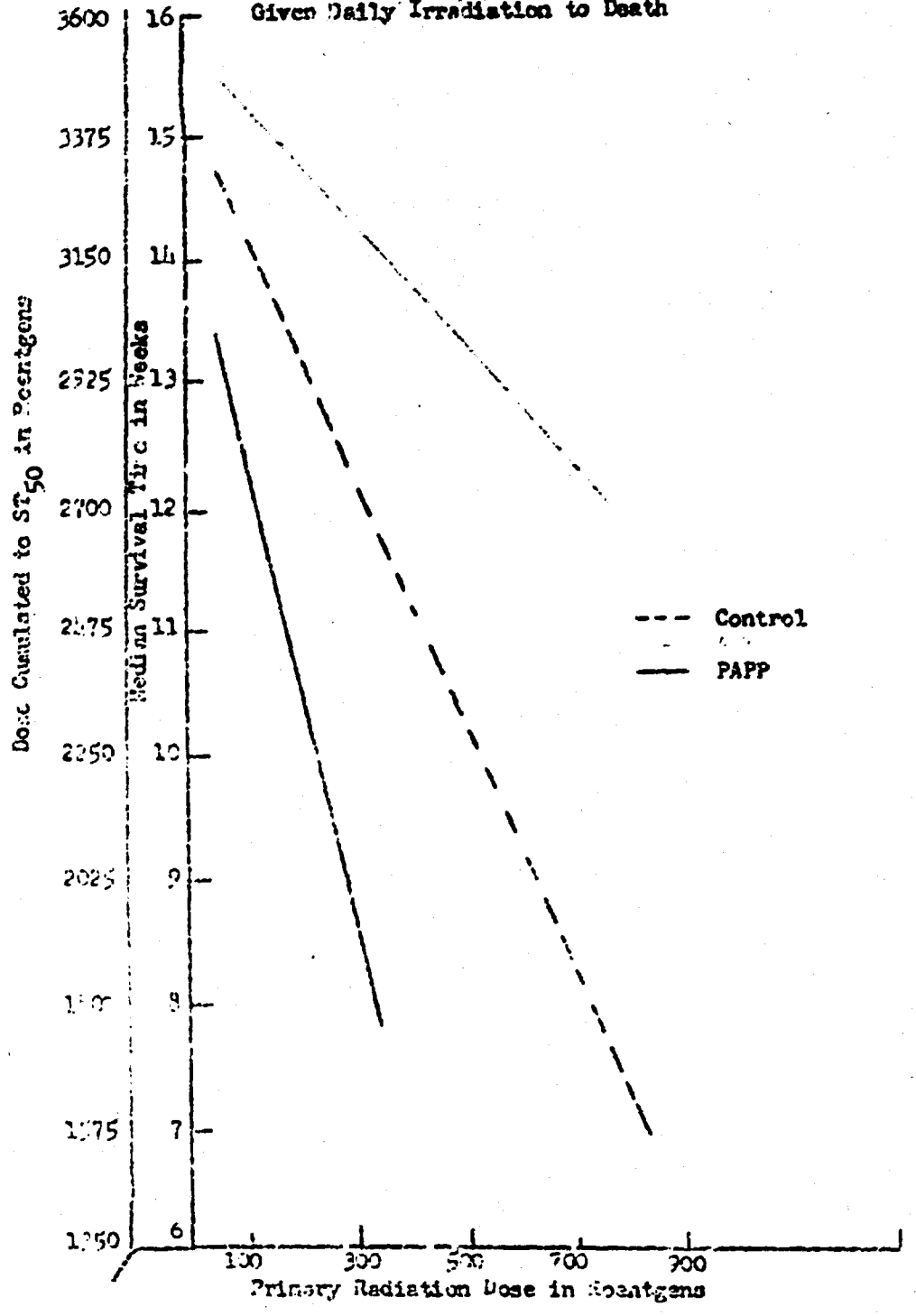
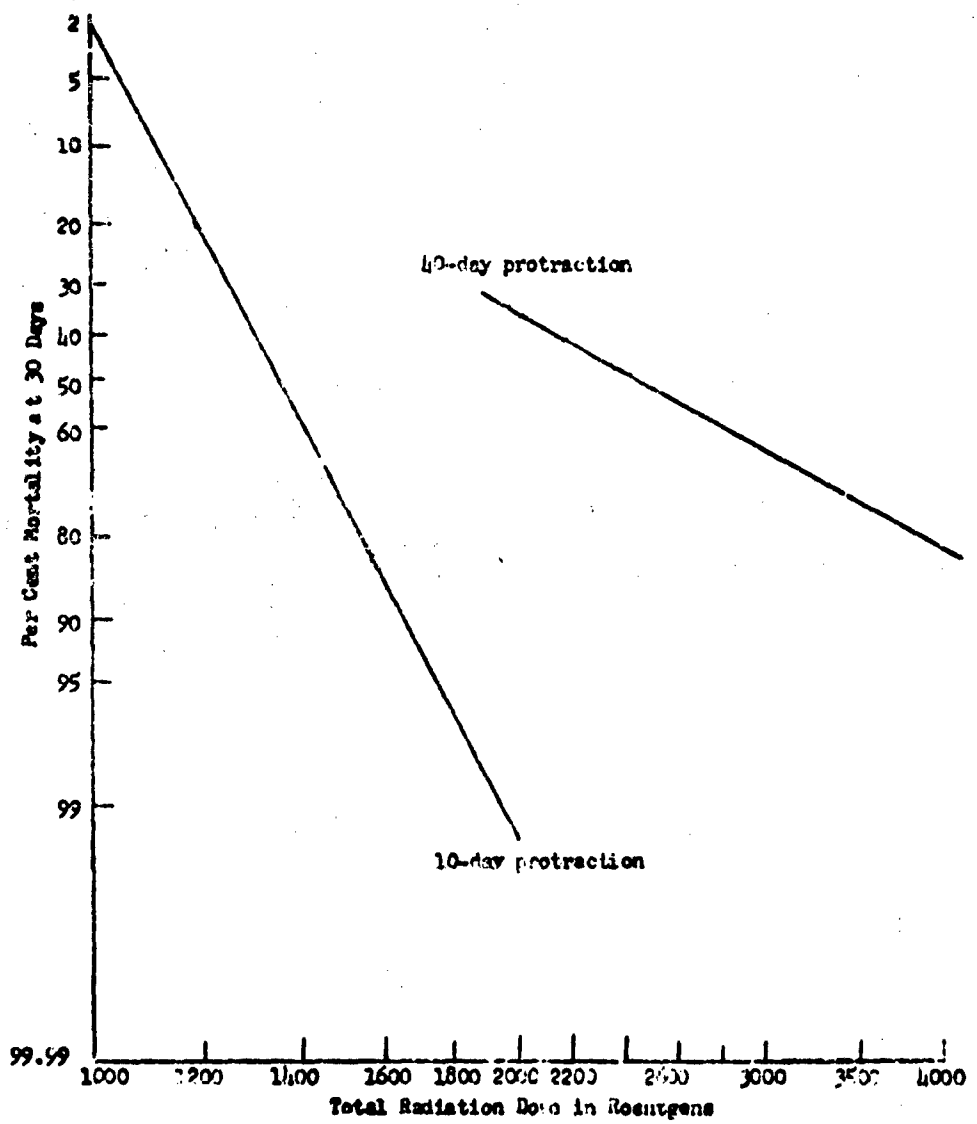


Figure 7

Effect of Protraction on the LD_{50} (30-Day) of Mice Exposed to Various Doses of Whole Body Gamma Irradiation



forty days and the subsequent mortality within this group determined during the 30-day period following the last radiation exposure. When the radiation was protracted over [illegible] of forty days, the LD_{50} dose was found to be increased to about 2[illegible] third series is currently in progress in which the protraction period [illegible]nty days. Previous studies have indicated that the LD_{50} for single doses of whole body gamma irradiation under similar experimental conditions is about 750 rep. It is apparent, therefore, that the effect of the protraction of the radiation exposure can be quantitated in terms of the protraction interval and that these results can be used to determine whether the protraction effects resulting from the daily radiation to death procedure are similar

Discussion

It is generally concluded that following exposure to either acute or chronic radiation injury in mammals, there is a residue of injury which is independent of time and persists throughout the subsequent life of the animals resulting in a different mean physiological state in the irradiated than in the control animals. Since mortality can be considered to be an expression of the mean physiological state (4,5), then any function which provides a linear expression of population survival should be displaced by a dose-dependent amount which would be equivalent to the irreparable damage from the radiation exposure. It must be assumed in applying this theory that every increment produces an effect that is independent of other increments so that injury from more than one exposure is expressed by the sum of the residual injury from each individual exposure. When the exposures are given sufficiently close together, the response will be dependent on all of the dose increments and on the time intervals between increments. Shortening of the mean life span as a late effect of radiation exposure to dosage levels which fail to cause acute mortality is widely used as a means for evaluating the residual damage from both acute and chronic radiation. Such studies are time-consuming, however, because of the long latent period between the radiation exposure and the development of the late effects. The split dose technique provides a method for estimating the residual damage without waiting for the development of the late effects. As generally used this procedure attempts to evaluate the residual damage by measuring the amount of additional radiation required at various intervals after the original radiation exposure to produce mortality (acute death). This technique has been used by several investigators (6,7,8,9) to obtain information on residual injury from single and fractionated radiation exposure and to calculate recovery rates. In this type of study the measurement of the additional radiation required for acute death is often made by determining the LD_{50} of the irradiated animals during the latent period. Since this requires rather large groups of animals for precise estimation, it appeared to us that the same sort of information could be obtained with fewer mice by giving the animals daily radiation to death rather than single radiation doses and using the median survival time as the response rather than the LD_{50} values. Preliminary studies (1,2) demonstrated that the daily dose procedure can be used to evaluate the life shortening effect of various doses of radiation and that the method is sensitive enough to provide information on the effects of fractionation and protraction of the radiation exposure.

It was of interest, therefore, to determine whether this technique would also be useful in providing information on the ability of radioprotective agents

to prevent the late effects of radiation exposure (assuming that life shortening is indicative of such effects). The results of these studies indicated that the radioprotective agents, p-aminopropiophenone and hydroxyacetonitrile, not only failed to prevent the life shortening in chronically irradiated animals but actually increased their mortality rate. 2-Aminoethylisothiuronium appeared, on the other hand, to prolong somewhat the survival time of the mice under similar experimental conditions. Radioprotective effects against acute radiation injury (as evaluated by the 30-day mortality following the single or primary exposure) were evident with all three of these agents. It should be emphasized that these studies are preliminary in nature. Additional groups of mice are currently in progress which will provide information over a wider range of primary radiation dosage levels and at several of the intermediate dosage levels not tested in this study. Several additional control groups have also been added to the study so that calculation of the per cent reduction in median survival time can be made and used as the basis for evaluating the radioprotective effects. One additional control group of mice which has been added as the result of the present study consists of mice given the protective agents but no primary radiation dosage. The results of the present study suggest that the protective agents may be exerting some effects independent of those influencing the primary radiation exposure. If the additional studies in progress support this observation, this would indicate the radioprotective agents may exert chronic or delayed actions as well as acute effects and would be of considerable practical and theoretical interest.

Summary

1. Studies have been initiated to determine whether the more common radioprotective agents are capable of protecing animals against the long term effects of radiation exposure (life span shortening) by means of a modification of the split dose technique.

2. Single whole-body radiation exposure in the range of 100 to 700 rep of gamma irradiation produce a linear shortening in the median survival time of mice given daily radiation to death at a dose rate of 225 rep/week.

3. The administration of p-aminopropiophenone prior to the primary or test dose of gamma irradiation does not protect the treated mice against the life shortening produced by a subsequent period of daily radiation to death.

4. There was no indication that the pre-irradiation administration of hydroxyacetonitrile was capable of preventing the life shortening effect of radiation exposure although significant protective activity against the acute radiation lethality (30-day) was evident.

5. There was a small but significant prolongation of the survival time of mice treated with 2-aminoethylisothiuronium prior to primary radiation exposures of 100 through 700 rep and subsequently given daily irradiation to death. Additional studies which are currently in progress will be required to evaluate the magnitude of this effect.

6. Studies are also in progress to investigate the effects of fractionation and protraction on the life span of irradiated animals using the daily radiation to death technique.

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THE INFLUENCE OF EXPOSURE TO LOW LEVELS OF GAMMA AND FAST NEUTRON IRRADIATION ON THE LIFE SPAN OF MICE

II. Preliminary Histopathological Examination of the Tissues of Mice Exposed to Fractionated Fast-Neutron Irradiation

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This report concerns: Histopathological findings in the tissues of male LAF_1 mice exposed to chronic fractionated doses of fast neutron irradiation at dosage levels of 19 rep to 737 rep.

Immediate or ultimate application of the results: To provide comparative information concerning the relative biological effects resulting from chronic exposure to fractionated gamma and fast-neutron irradiation. Information of this type should be of value in establishing the actual and relative biological hazard associated with time-limited exposure to different types of ionizing radiation.

* * * * * * * * *

In initiating the gamma-fast neutron irradiation program of this laboratory the primary purpose was to obtain information on the comparative effects of these two types of radiation on the life span of animals. Thus only those tests which did not injure the animals were carried out. The results of these studies have been summarized in previous reports (1,2,3). During the course of these studies, animals which died were autopsied whenever possible and the tissues prepared for histological examination.

The present report contains the results of our preliminary examinations of the tissues from the mice which were exposed to various doses of fractionated fast-neutron irradiation. In subsequent studies, similar examinations will be made on the tissues of the mice which were exposed to comparable doses of gamma irradiation. It is anticipated that these studies will permit us to evaluate the relative biological effectiveness (RBE) of the two types of radiation exposure in relation to the pathological changes produced and that the RBE values thus obtained will supplement the previous RBE values determined in relationship to development of cataracts and the shortening of the life span of the animals.

Materials and Methods. Detailed descriptions of the gamma and fast neutron irradiation facilities together with information concerning the calibration techniques have appeared in previous reports (4,5). The experimental design of the irradiation programs and the various patterns of fractionation and protraction employed have also been described (6).

Over 1,200 male LAF_1 mice were employed for the fast neutron irradiation studies. Tissues from about 10% of these animals have been examined in

the present study. Mice representing each of the seven experimental groups (radiation doses of 0 rep to 737 rep) were included. In Table 1 is shown the radiation dosage received by each of the groups, the number of animals examined from each group and the number of days after irradiation at which death occurred or in the case of the control group at which the animals were sacrificed. The age of the individual animals at the time of death and the distribution of the major pathology can be found in Appendix I of this report. The radiation exposures to which the mice in this study were subjected were given at dosage rates of 1/15 rep, 1/5 rep or 3/5 rep per hour and the animals were irradiated for periods of 1/12, 1/4 and 3/4 of each of the radiation cycles which consisted of 1-day, 3-day and 9-day intervals. The radiation exposures were protracted over total periods of 18 days, 54 days or 162 days. By using a latin square experimental design for the radiation program, it was possible to combine several of the groups in order to investigate in greater detail the effects of variation in dose rate, fractionation, protraction and total dosage. In the present study we have limited the comparisons to the total dose since this provides the best first approximation of the radiation effects. It is anticipated that some additional information may be obtained in further studies regarding the effects of fractionation, protraction and dose rate, particularly in the gamma-irradiated groups since there is a much larger population of gamma irradiated mice.

TABLE 1

RADIATION DOSE, NUMBER AND AGE OF ANIMALS AND GROUP NUMBER OF MICE EXAMINED IN THIS STUDY

Group Number	Total Fast Neutron Dose	Number of Animals Examined	Age at the Time of Death or Sacrifice (Days)
I	Control	17	140-736
II	19 rep	13	52-736
III	57 rep	15	294-580
IV	170 rep	35	26-571
V	510 rep	25	147-572
VI	737 rep	5	193-314
VII	To death (700 to 1,324)	7	154-323

The following organs were removed, weighed and prepared for histopathological examination: brain, eye, heart, lung, liver, spleen, lymph nodes (cervical and mesenteric), kidneys, adrenals, thymus, testis, urinary bladder, stomach, duodenum, ileum, colon, submaxillary salivary gland and bone marrow from the sternum and from the femur. Not all of these tissues were obtained from each of the animals. The thymus, for example, was taken rather infrequently in the irradiated animals.

Neutral buffered formalin was used routinely as the fixative for the tissues which were then imbedded in paraffin and stained with hematoxylin and

eosin. In addition, some of the tissues were also stained with special stains (congo-red and gentian violet) to demonstrate the presence of amyloid.

Experimental

Group I. Control Animals, Male LAC, Mice

This group contained seventeen animals. The animals were killed periodically in order to compare the tissues at different ages which ranged from 140 to 736 days.

Histopathological Findings in Different Organs

Lung. Four out of seventeen animals had tumors in the lung. One animal had a pulmonary adenoma (U-384), two had lymphomas and one had leukemia. Slight to moderate accumulation of chronic inflammatory cells was observed in peribronchial and perivascular region in eight animals. Slight anthracosis in two, atelectasis in one, and thickening of alveolar wall with minute hemorrhages were observed in another animal.

Liver. Five animals showed some focal accumulation of chronic inflammatory cells mostly in periportal region. Two had slight proliferation of Kupffer cells. The cytoplasm of the hepatic cells was vacuolated in two mice. Leukemia was observed in two animals. Only one animal had deposition of homogeneous slightly eosinophilic substance, probably amyloid, which infiltrated and surrounded the blood vessels particularly in the portal region. The age of this animal was 677 days.

Spleen. In the spleen of three animals lymphoma was observed. Two animals had extramedullary hematopoiesis and hemosiderosis. Amyloidosis was observed in the same animal which had amyloidosis in the liver, replacing diffusely nearly all splenic tissue.

Kidney. Small foci of chronic inflammatory cells were observed in eight animals. Slight leukemic infiltration was present only in one animal. Amyloidosis was observed in one animal.

Lymph node. One animal showed extramedullary hematopoiesis and slight hemosiderosis.

Adrenals. Amyloidosis was also observed in adrenals and stomach of the same animal that had similar changes in spleen, kidneys and liver.

Group II. Virgin, Male LAC, Mice

In this group we had [illegible] animals varying in age from 52 to 736 days.

Histopathological Findings in Different Organs

Heart. Leukemic infiltration was observed in the brown fat in the vicinity of the heart of one animal.

Lung. Leukemia was observed in the lungs of five animals. Atelectasis of the lung was present in five animals. Two animals had anthracosis and three animals had hyperemia.

Liver. In the liver leukemic infiltrates were observed in nine animals. Moderate proliferation of inflammatory cells in periportal region was present in two animals. Variation in size and shape of nuclei was observed in one animal. These nuclei appeared also pale and vacuolar. Eight animals had amyloidosis of varying degree. In most of them the periportal region was involved but in a few at least half of the liver tissue has been replaced by amyloid due to spreading of amyloid deposition from subendothelial region in the portal vein and along the hepatic sinusoides, causing atrophy of liver cells.

Spleen. Hemosiderosis and extramedullary hematopoiesis were present in the spleens of three mice. Seven mice had leukemia. Amyloid was present in the spleen of six animals. The sites of preference seem to be perifollicular venous sinuses, sometimes surrounding follicles completely, or entirely replacing red pulp. In one case nearly the whole spleen tissue was replaced, leaving only small nests of leukemic cells.

Lymph node. Leukemia was observed in one lymph node.

Kidney. Lymphoid leukemic infiltrates were present in the kidneys of seven animals. Perivascular infiltration of lymphoid tissue was observed in five other mice. Dilatation of renal tubules filled with albuminous fluid was observed in four animals, one of which had actual cyst formation in the cortex of the kidney. Amyloidosis was observed in one animal involving mostly glomeruli (giving a somewhat nodular appearance) and the walls of the renal blood vessel. There was also slight deposit in the interstitial tissue.

Adrenals. Adrenals were another region where amyloid was deposited. It was seen in four animals; one of them had nearly the whole gland replaced by amyloid. One animal had spotty distribution of amyloid involving medulla and the other two had only cortex involved (particularly zona reticularis). The animal with most pronounced amyloidosis had also slight leukemic infiltration.

Testes. Slight atrophic changes were observed in seminiferous tubules of two mice. They appeared disorganized and there was disappearance of germinal epithelium.

Thymus. Only two thymic samples were available. One animal had slight deposition of amyloid in the thymus and in the other animal the thymus appeared very atrophic.

Urinary bladder. Slight infiltration of lymphoid tissue in perivascular region was observed in the urinary bladder of one mouse.

Gastrointestinal tract. Hyperplasia of mucosal epithelium with slight irregularity of height and width of the villi was found in six animals. Lymphoma was observed only in the stomach and duodenum of one animal. Lamina propria and submucosa of duodenum and ileum contained amyloid deposition in one mouse.

Bone marrow. Three animals showed leukemic changes in the bone marrow. Bone marrow of the femur in one animal had acellularity in metaphysis and two had much vascularity in the epiphyses.

Tumors. One of the animals had two tumors in the abdominal cavity both in vicinity of the kidneys. Histopathological examination revealed that they were lymphomas.

Group III, 57 rep, Male (AF_1) Mice

This group had fifteen animals for histopathological examinations varying from 294 to 580 days.

Histopathological Findings in
Different Organs

Lungs. Lymphoma was observed in five animals. In one case it appeared that secondary infection with gangrenous necrosis was superimposed on the leukemia. Five mice had peribronchial proliferation of lymphoid tissue. Atelectasis was observed in six animals. Anthracosis was seen in two animals. Hyperemia appeared in one of these two mice. Hyperplasia of bronchial epithelium was present in two animals.

Liver. The most pronounced change observed in the liver was deposition of amyloid. Nine of fifteen animals had it, replacing in the majority of cases two-thirds of the liver tissue. Deposition originated most frequently in periportal region extending through sinusoids and reaching the central vein. In some animals the amyloid had a crystalloid aspect. Leukemia was observed in five animals. Two mice had extramedullary hematopoiesis, myeloid in nature. Eosinophilic intranuclear inclusion bodies were seen in one animal. In seven animals irregularity in size and shape of the nuclei was seen. In one animal accumulation of inflammatory cells was observed in the portal region.

Spleen. A frequent finding in this organ also was development of amyloidosis. It was present in ten animals. The site of greatest involvement was the perifollicular venous sinuses. In six spleens the red pulp was entirely replaced by amyloid. Leukemia was present in seven animals. Eight animals had extramedullary hematopoiesis. Hemosiderosis was seen in five animals.

Lymph node. In one animal the cervical and mesenteric lymph nodes were hemorrhagic and amyloidosis in the form of little nodules was present in mesenteric lymph nodes.

Kidney. Leukemia was present in four animals. Perivascular infiltration of lymphoid tissue was observed in six animals. Three animals had amyloid

deposition in the wall of renal blood vessels. In this group eight animals had dilatation of renal tubules and cyst formation. The dilated tubules and cysts contained albuminous fluid in lesser or larger amounts.

Adrenals. Deposition of amyloid was observed in nine animals. The zona reticularis or cortex in general appeared to be most involved but in two animals the medulla was also involved. In one mouse amyloid was diffusely distributed leaving only small patches of cortical and medullar tissue intact. Vacuolisation of the cells of the cortex was observed in one animal. Slight leukemic infiltration was present in one mouse. Hyperplastic adrenal glands were observed in one mouse.

Testis. In one mouse a few seminiferous tubules did not have visible spermatogenesis.

Thymus. Leukemia was seen in one of the two thymus glands sampled in this group.

Gastrointestinal tract. In seven animals hyperplasia of mucosal epithelium was observed in the small intestine. One animal had a slight increase of lymphoid tissue in the lamina propria. Amyloidosis in the lamina propria was seen in two mice.

Bone marrow. Leukemia of the bone marrow was observed in six animals.

Eye. Iridocyclitis and retinitis was observed in the eye of one mouse.

Group IV, 170 rep, Male LAF_1 Mice

In this group there were thirty-five animals with their age varying from 26 to 571 days.

Histopathological Findings in Different Organs

Lung. The most common finding was atelectasis observed in sixty animals, thirteen of which had concurrent thickening of the alveolar wall characterized by swelling of cuboidal cells. Leukemia was present in eleven animals. Peribronchial accumulation of lymphoid tissue was seen in nine mice. In one of them lymphoid tissue nodules were found. A pronounced hyperemia was present in three animals. Three pulmonary adenomas were also seen. Proliferation and swelling of cells of alveolar linings together with hyperemia. One animal had interstitial pneumonia.

Liver. In this group nineteen animals had deposition of amyloid. In most of them one-third of the liver tissue was replaced by it and in two mice only a small part of the liver tissue was spared. In eighteen animals leukemia was observed. Two mice had slight leukemic infiltration. Another frequent finding was irregularity in size and shape of nuclei. In a few animals giant-size nuclei were observed. Four mice had intranuclear inclusion bodies and in one of them cytoplasmic inclusion bodies were present too. Focal nuclear

hyperchromatism was observed in a few animals. Vacuolisation of the cytoplasm and nucleus was seen in three animals. Two animals had coagulation necrosis, in one of them an abscess was also seen. Perivascular accumulation of inflammatory cells was present in four animals.

Spleen. Most frequent findings in the spleen was deposition of amyloid. It was seen in twenty-two animals. Amyloid, as in previous groups, was mostly seen in the perifollicular region or in some cases replacing the red pulp completely. In one mouse patchy nodular distribution was observed. Leukemia was observed in eighteen animals. Haemosiderosis was present in seventeen and extramedullary haematopoiesis in twelve animals.

Kidney. Leukemia was present in eight animals. Four animals had amyloidosis most frequently observed in glomeruli, blood vessel walls and some times in the interstitium. Dilatation of the tubules and cyst formation was seen in twelve animals. Mostly these spaces were filled with albuminous fluid, which varied from slightly to markedly eosinophilic. Abscesses were seen in two animals and one animal had pus in the tubules. Thirteen animals had lymphoma.

Lymph node. In two animals leukemia was observed. Haemosiderosis was observed in one animal together with slight leukemic infiltration. One lymph node had amyloid deposition together with fibrosis.

Adrenals. In seven mice amyloidosis was observed. The site of predilection seem to be the cortex, but in two animals both cortex and medulla were involved leaving only a narrow strip of adrenal tissue in the cortex. Patchy distribution of amyloid was seen in one of the mice.

Testis. The seminiferous tubules showed focal atrophy in six animals. In two animals seminiferous tubules were filled with eosinophilic material. One mouse had a few tubules distorted, dilated and filled with blood. One testis had a tumorous hemorrhagic mass replacing most of the testis.

Group V, 510 rep, Male IAF$_1$ Mice

In this group there were twenty-five animals between 147 to 572 days old.

Histopathological Findings in
Various Organs

Heart. In one mouse some inflammatory cell infiltration was present.

Lung. Atelectasis was observed in nine animals. Leukemia was present in six mice. One mouse had metastatic hepatoma in lung together with leukemic infiltration. One animal had a pulmonary adenoma. Bronchopneumonia was present in three animals. A pronounced hyperemia was observed in two mice. One animal had anthracosis. Slight hyperplasia of bronchial epithelium was observed in three mice.

Liver. Leukemia was present in six animals. Hepatoma was observed in two animals and adenocarcinoma in one mouse. Amyloid deposition was present in nine animals. Necrosis in the liver probably due to infection was observed in six animals. In some of them it appeared to be chronic. The amount of connective tissue was increased, the leukocytic inflammatory response was less and the architecture was distorted in the periportal region. Degenerative changes with vacuolization of cytoplasm were present in four animals. Accumulation of inflammatory cells in the portal region was observed in four animals. Three mice had intranuclear inclusion bodies. Two mice had extramedullary hematopoiesis of myeloid origin. A frequent finding in this organ was irregularity in size and shape of nuclei accompanied sometimes with bizarre hepatic cells. One mouse had cholangitis.

Spleen. Hemosiderosis was present in thirteen animals. Extramedullary hematopoiesis was observed in nine mice. One mouse had slight fibrosis. Six animals had leukemia. Hyperemia was observed in one animal. Amyloid deposit was present in ten animals, in few of them replacing half and more of splenic tissue.

Lymph node. Two mice had leukemia and in the other two hyperemia was observed.

Kidney. Amyloidosis in the glomeruli was seen in seven animals. Leukemia was present in two and slight leukemic infiltration in two other mice. Pyelonephritis was observed in two animals with superimposed necrosis. Leukemic infiltration was seen in four animals. Focal vacuolar parenchymatous degeneration was observed in one mouse.

Adrenal. Three animals had amyloid deposition located mostly in zona reticulosis. Formation of small cyst-like spaces in zona fasciculata was observed. One adrenal had leukemic infiltrate. Hemorrhage was present in one gland. One gland was hyperplastic.

Thymus. Three glands out of four sampled had leukemic involvement.

Testis. In sixteen animals slight to moderate degenerative changes of seminiferous tubules were observed. In one mouse amyloid deposit was present in interstitial tissue and in the wall of some blood vessels.

Urinary bladder. Desquamation and proliferation of transitional epithelium was observed in five animals.

Gastrointestinal tract. In one of the animals ulceration of the stomach was observed. Hyperplasia of mucosal epithelium was present in one animal in the stomach and duodenum. Amyloidosis in lamina propria was seen in one mouse. Irregularity in width and height of villi in the duodenum was observed in four animals.

Bone marrow. In two animals the bone marrow appeared very active. In three of them it was somewhat hypocellular and vascular.

Group VI. 737 rep. Male LAF_1 Mice

In this group there are five animals. Their age varies between 193 to 311 days.

Histopathological Findings in Various Organs

Brain. Disappearance of several Purkinji cells was observed in one mouse.

Lung. Proliferation of bronchial epithelium was present in three animals. Early leukemic infiltration in peribronchial region was seen in three mice. Hemorrhages were observed in two animals. One animal appeared to have viral pneumonia which was characterised by an inflammatory reaction localised within the walls of the alveoli. Besides edema there was mononuclear inflammatory cell infiltration in the alveolar septa. Amyloid deposition in the blood vessel wall was observed in one mouse.

Liver. Leukemic infiltration was present in three mice. Amyloidosis was observed in three animals. Patchy necrosis was seen in two animals, in one being quite prominent. The cytoplasm of hepatic cells showed slight vacuolisation in two mice. Eosinophilic intranuclear inclusion bodies were observed in one mouse.

Spleen. Extramedullary hematopoiesis and hemosiderosis was observed in five animals. Amyloid deposition was present in three animals involving partly red pulp. Leukemic infiltration was present in three animals.

Kidney. In two mice leukemic infiltration was observed. Hypercellularity of glomeruli was seen in one mouse. One focal granuloma was present in another animal.

Adrenals. An adenoma was observed in one of the animals. Amyloidosis was present in three animals mostly involving the cortex, but in one mouse the medulla was involved too. Vacuolisation of the cytoplasm of medullary cells was observed in one mouse.

Testis. In five animals degenerative changes of seminiferous tubules were observed. In three cases only Sertoli cells were left, sometimes this appeared in more than half of the seminiferous tubules.

Gastrointestinal tract. In one mouse amyloid deposit was observed in the duodenum giving to the villi a "club-like" appearance. Irregularity in size and shape of the villi was observed in two animals. Mild proliferation of epithelium of mucosa in the colon was observed in two mice.

Bone marrow. In two animals apparent acellularity together with increase vascularity was observed. Two mice had hyperplastic marrow with myeloblastic elements prominent.

Group VII, No death, Male LAF1 Mice

In this group there are seven animals. Their age varies from 154 to 183 days.

Histopathological Findings in Various Organs

Brain. Disappearance of several Purkinji cells was observed in one animal.

Lung. One animal had pulmonary adenoma. Proliferation of bronchial epithelium was present in three animals. Atelectasis was seen in two animals. Hemorrhages in the alveolar wall and space was observed in one animal. The lungs of one mouse had anthracosis, and three animals had early leukemic infiltration.

Liver. Irregularity in size and shape of nuclei was observed in three animals. Vacuolization of nuclei was seen in one mouse. Leukemia was present in one animal. The nuclei of one mouse had eosinophilic inclusion bodies. Amyloid deposition was observed in one animal and proliferation of Kupffer cells in another.

Spleen. Hemosiderosis was present in six animals. Leukemia was observed in three animals. Extramedullary hematopoiesis of the myeloid type was present in two animals. One system had its red pulp replaced by amyloid.

Lymph node. Leukemia was observed in one animal. This was the only lymph node sampled in this group.

Kidney. In one animal leukemic infiltration was present.

Adrenal. Two adenomas of the adrenal cortex were seen. One of them had slight leukemic infiltration. One mouse had amyloid deposited in the medulla and zona reticularis of the cortex.

Testis. Atrophy of seminiferous tubules was observed in five animals. The degree of atrophy varied in some of them involving only a few seminiferous tubules, in others the whole testes had undergone necrosis or only "ghost-like" tubules were left.

Gastrointestinal tract. Ulceration was present in the stomach of one mouse. In seven animals slight hyperplasia of mucosal epithelium was present in the small intestine. Irregularity of the villi was observed in four animals. Slight increase of mononuclear inflammatory cells was observed in the stomach of two and the duodenum of one animal.

Bone marrow. Increased vascularity was present in one animal.

Tumor. One animal had a tumor in lumbosacral region. It appeared to be squamous cell carcinoma.

Discussion

The histopathological observations presented in this report represent a preliminary effort to evaluate the magnitude of radiation injury resulting from chronic exposure to fractionated fast neutron irradiation in mice. When similar information is obtained on gamma irradiated mice under similar experimental conditions of dose rate, fractionation and protraction, it should be possible to compare the type and severity of histologic injury produced by the two types of radiation.

It is apparent from the studies presented in this report that fractionated fast-neutron irradiation is associated with an increased incidence of amyloidosis and of leukemia and other neoplasms. In general, the frequency of these conditions is greater with increasing radiation dosage although a linear dose-response relationship was not obtained. A more detailed analysis of the frequency data in terms of the age of the animals, the radiation parameters and the type of histological change may improve the dose-response relationships and studies of this type are now in progress. These studies will be facilitated in the analysis of the data from the gamma-irradiated mice because of the much larger population in each of the various radiation parameters.

Leukemia as a late effect of radiation exposure is a well-established finding in radiobiology. Information is needed, however, on the effects of fractionation and protraction of the radiation exposure on the incidence, time of onset and rate of progression of this condition. The incidence of leukemia and other neoplasms observed in the present studies is summarized in Table 2. Here it can be seen that leukemia was the most frequent finding in both the control and experimental groups. The highest incidence was observed in mice which received a total radiation dose of 737 rep of fast neutron irradiation. There was a greater incidence of leukemia in the irradiated groups than in the control animals although nearly half of the control mice exhibited leukemia. Lung adenomas were found in three of the irradiated groups and were also present in the control mice (6%). Two of the irradiated mice had hepatomas and irradiated animals with testicular tumors and a squamous cell carcinoma were noted. None of these tumors were detected in the control animals although it must be recognized that the number of animals involved is small. In Table 3 is shown the incidence of multiple primary neoplasms in these animals and it is apparent that only a few of the animals developed two primary neoplasms. None of the mice examined in these studies developed three or more primary tumors. The organ distribution of leukemia is of interest in that leukemia was detected only in the liver, spleen and lungs of the control mice whereas the irradiated group also exhibited this disease in the kidneys, lymph nodes, gastrointestinal tract and occasionally in other organs.

The second most frequent pathologic finding in the tissues of these mice was amyloidosis. Although about 6% of the control mice exhibited this condition, the incidence was greatly increased (over 50%) in all of the irradiated groups. Thus in the mice which received the lowest total radiation exposure (19 rep) ten out of thirteen animals had these changes in one or more organs. Only one animal in the control group exhibited amyloidosis and the latent period in this animal was nearly six hundred days. In the irradiated mice the latent period was reduced and many of the irradiated mice exhibited

TABLE 2

INCIDENCE OF LEUKEMIAS AND OTHER NEOPLASMS

Group	No. of Mice	Total Irradiation Dose	Response							
			Leukemia		Lung Adenoma		Hepatoma		Various	
			No.	%	No.	%	No.	%	No.	%
I	17	0	7	41.17	1	5.87	0	0.00	0	0.0
II	13	19 rep	9	69.23	0	0.00	0	0.00	...	...
III	15	57 rep	11	73.32	0	0.00	0	0.00	...	....
IV	35	170 rep	23	65.71	3	8.57	0	0.00	1[a]	2.8
V	25	510 rep	10	40.00	1	4.00	2	8.00	0	...
VI	5	737 rep	4	80.00	0	0.00	0	0.00	0	0.0
VII	7	No death	3	42.85	1	14.28	0	0.00	1[b]	28.5

[a] Tumor of testis.

[b] Squamous cell carcinoma.

TABLE 3

INCIDENCE OF TWO MULTIPLE PRIMARY NEOPLASM IN INDIVIDUAL MICE

Group	Total Number of Mice	Number of Mice with Two Primary Neoplasms
I	17	1
II	13	1
III	15	1
IV	35	6
V	25	3
VI	5	1
VII	7	2

the disease within two hundred days after the initial radiation exposure. The organ most frequently involved was the spleen and the incidence decreased in the following order: liver, adrenal glands, small intestine, kidney, lymph nodes, thymus and lungs. Tsung (1) has suggested that senility in mice causes amyloidosis in all organs with the spleen and liver being the least involved in old animals. The spleen and liver are common sites of amyloidosis induced experimentally in mice and it may be that the radiation exposure is acting more as a stressing factor than by causing premature ageing in these studies. These results are summarized in Table 4. It is of interest to note that organ involvement by amyloidosis in neutron irradiated animals was very much the same as found by Ting-Ma Wong et al. (1) in their study with Tween 80 and methylcholanthrone.

Variation in the shape and size of the nuclei and cells as well as abnormal mitosis were seen in the livers of the irradiated mice. This was particularly marked in the animals in groups II, III, IV and VI. The size of the hepatic nuclei in these animals varied widely within the same section and the giant nuclei were frequently hyperchromatic. Abnormal shaped nuclei (dumbell shaped, polycyclic borders, etc.) may have been due to virus infection since there were eosinophilic intranuclear inclusion bodies present in the livers of the animals of groups IV and VI and possibly in the other groups. The possibility exists, of course, that this viral infection was easier established by the toxic effect on the liver cells which altered in some manner the susceptibility of the mice to the virus. There was also an increase in the connective tissue in the portal region of the liver of the irradiated mice. Infiltration of inflammatory cells, alteration of liver architecture and slight necrosis was frequently seen in irradiated animals. These effects were more prevalent in the livers of the younger age groups within the irradiated animals and were not noted in the livers of the control animals in the same age group.

Hyperplasia of the mucosal epithelium and irregularity of the villi were commonly seen in the small intestine of the irradiated mice and is assumed to be due to the regeneration of the radiation damaged mucosa of these animals. These changes were not present in the intestines of the control mice.

The mice in groups V, VI and VII exhibited atrophy and other pathological changes in the testicular tubules although there was considerable variation in the severity of the changes. Many of the mice exhibited disorganization and disappearance of the germinal epithelium of the testis without apparent injury of the sertolli cells. Primary spermatocytes and intact sperm were observed in the tubules in some of these animals. A few of the mice exhibited only shrunken tubules consisting of a single row of cells of indefinite nature filled with eosinophilic amorphous material. This probably represented a mixture of degenerated cells and syncitium of the Sertoli cells. Similar findings have been reported by Ely et al. (3) in neutron-irradiated mice. The testicular changes observed in the irradiated animals appeared to be correlated to some degree with the total radiation exposure.

Extramedullary hematopoiesis was frequently observed in the spleen and was common in the liver of the irradiated and control mice. In the spleen, from 25 to 100% of the animals had this finding and it did not seem to be consistently correlated with the radiation dose. Since this represents a variable

TABLE 4

TOTAL INCIDENCE OF AMYLOIDOSIS PER GROUP PER ORGAN

Group	Number of Mice Examined	Total Irradiation Dose in rep	Response						
			Total Number of Mice Showing Amyloidosis	Per Cent	Number of Mice with Number of Organs Involved				
					1 Organ	2 Organs	3 Organs	4 Organs	5 Organs
I	17	0	1	5.87					1
II	13	69	10	76.92	1	6	2	1	
III	15	137	13	86.66	1	6	3	2	1
IV	35	170	24	68.57	2	7	10	4	1
V	25	510	14	56.00	5	3	5	0	1
VI	5	737	4	80.00	1		2	1	
VII	7	To death	1	14.28			1		

finding even in the control animals, no pathologic significance can be ascribed to the occurrence of this condition in the irradiated animals at present.

Many of the pathologic findings described in this report are consistent with the hypothesis that radiation accelerates ageing. The increased incidence of amyloidosis, the induction of a variety of neoplasms and the progressive impairment of the reproductive organs, for example, lend support to this concept. The organ distribution of the changes particularly amyloidosis and the type of neoplasms detected are not in complete accord with the ageing concept. It is apparent that additional studies will be required to provide greater insight into this question and it is hoped that the examination of the tissues from the gamma-irradiated mice may be helpful in this respect.

Summary

1. Histopathologic examination of the tissues of LAF_1 male mice chronically exposed to fractionated fast neutron irradiation at dosage levels of 19 rep to 737 rep have been carried out using a total of one hundred irradiated mice and seventeen non-irradiated control animals.

2. Leukemia was the most frequent pathologic finding in both the control and irradiated mice and over half of the animals examined exhibited this disease. The number of organs involved and the incidence of the disease was greater in the fast-neutron irradiated mice and was to some extent related to the total dose of radiation administered.

3. Amyloidosis was present in about 6% of the control animals and was markedly increased at all of the radiation dosage levels employed. The latent period for the development of this condition was shortened in the irradiated mice and the severity of the disease was more marked in the irradiated animals.

4. Lung adenomas were found in three of the irradiated mice. Hepatomas were present in two animals and mice with testicular tumors and a squamous cell carcinoma were detected among the irradiated groups. One lung adenoma was observed in the tissues of the control animals.

5. The fast neutron irradiated mice exhibited degenerative changes in the tubules of the testis which were characterized by atrophy and disappearance of germinal epithelium. Spermatogenesis was present in the testis of some of the irradiated animals.

6. Changes in the size and shape of the liver cells were observed in the irradiated mice which were suggestive of a viral infection. These changes were not present in the liver of the control animals, however, which would suggest that the radiation exposure in some manner altered the susceptibility of the mice to the viral infection.

7. Hyperplasia of the mucosal epithelium and irregularity of the villi were observed in the small intestines of the irradiated mice and are attributed to regeneration of the radiation injured mucosa since these effects did not occur in the control animals.

Refere ices

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APPENDIX I

In Appendix I incidence of amyloidosis and leukemia in mice chronically exposed to fractionated, fast-neutron irradiation is given. Besides dosage of irradiation expressed in rep, age of animals after initial irradiation exposure, organs involved and total number of animals in each group are also presented.

APPENDIX I

INCIDENCE OF AMYLOIDOSIS AND LEUKEMIA IN MICE CHRONICALLY EXPOSED TO FRACTIONATED FAST NEUTRON IRRADIATION

Group Number	Animal Number	Age at Death[a]	Liver	Spleen	Kidney	Lung	Adrenal Glands	GI tract	Other Tissues
I 0 rep	1	140							
	2	170							
	3	170							
	4	185		L					
	5	225							
	6	225							
	7	225							
	8	225							
	9	275				L			
	10	275							
	11	508				L			
	12	509							
	13	516	L						
	14	557	A L	A L	A		A	A	
	15	563				L			
	16	635		L					
	17	635							
II 19 rep	1	52							
	2	81							
	3	252	A				A		
	4	293	L						
	5	322	A L	A	A				L - lymph node
	6	341	A	A					
	7	346	A L	L	L	L			L - heart brown fat
	8	363	A L	A L		I			L - bone marrow
	9	374	A L	A L	L				L - bone marrow

[a] days after initial irradiation exposure. The animals were about 100 days of age at the time of their initial radiation dose.

A = amyloidosis; L = lymphoma.

APPENDIX I--Continued

Group Number	Animal Number	Age at Death[a]	Liver	Spleen	Kidney	Lung	Adrenal Glands	GI tract	Other Tissues
II 19 rep cont'd.	10	523	A L	A L	L		A		
	11	533	A	A			A		A - adrenals
	12	736	L	L	L	I	A	A	
	13	735	L	L	L	L	L	A L	L - adrenals
III 57 rep	1	294				L			L - tumor mass
	2	301	A L	A L	L	L	A		L - bone marrow
	3	317	A L	A L	L				L - bone marrow
	4	409							
	5	424	A		A				
	6	425	A L	A L			A		L - bone marrow
	7	427	A L		L		A		L - bone marrow
	8	428	A L	A					
	9	434	L				A		
	10	438		A L		L		A	L - bone marrow
	11	464		A L	L		A		L - bone marrow
	12	528	A	A	A		A		
	13	528		A	A	L	A		\ - lymph node mesenteric
	14	544	A	A L		L	A		L - bone marrow
	15	580	A	A			A	A	
IV 170 rep	1	26							
	2	28		A					
	3	96	L	L					
	4	121							
	5	121							
	6	284	A	A					L - bone marrow
	7	284							
	8	296	L		A	I		A	
	9	311		A				A	
	10	317	A L	A	L				

APPENDIX I--Continued

Group Number	Animal Number	Age at Death	Liver	Spleen	Kidney	Lung	Adrenal Glands	GI Tract	Other Tissues
	11	320	A L	A		L			Squamous carcinoma
	12	320	A	A				A	
	13	325							
	14	326	A L	A L	L	L		A	L - bone marrow
	15	348	A L	A L		L			
	16	355							
	17	355	A L	A L	L			A	
	18	359	A L	A L	A L				L - bone marrow
	19	359	A	A		L		A	
	20	362	A L	A L	A	L			
IV	21	366	A L	A					Tumor of testis
170 rep	22	378	A L	A L				A	
cont'd.	23	418	A L	A L	L		A	A	
	24	459	L	L		L			
	25	460	A L	A L	L		A		
	26	473	A L	A L		L	A	A	
	27	478	A	A		L			
	28	515	A L	A L			A	A	
	29	531		L		L			
	30	531		L					L - bone marrow
	31	551	A	A			A	A	
	32	570							A - lymph node
	33	583	L	L	L	L			L - bone marrow
	34	561	L	A L	L	L	A		L - bone marrow, lymph node
	35	571	A	A	A		A	A	
	1	117							
	2	217							
	3	237							
V	4	236		A L					L - thymus
510 rep	5	289							
	6	297	A						
	7	345	L						

APPENDIX I--Continued

Group Number	Animal Number	Age at Death	Liver	Spleen	Kidney	Lung	Adrenal Glands	GI Tract	Other Tissues
	8	348		A					
	9	355							
	10	355	A	A	A				
	11	362	L						
	12	364	A	A		L		A	
	13	357	A			L		A	
	14	379	A						
	15	382	A	A	A				
	16	384			A				
210 rad cont'd	17	399	A	A			A		
	18	429	L	L	L	L			
	19	472		A	A				
	20	473	A	A	A		A		A - testis
	21	530		A			A		
	22	513	L			L			
	23	517		L		L			L - lymph node; L - thymus
	24	563	L	L		L			L - adrenal
	25	572	L L	A	A				L - bone marrow, lymph node
	1	183		L			A		
VI 7.7 rad	2	220	A L	A			A	A	
	3	222							
	4	260	A L	A L	L		A		L - bone marrow
	5	314	A L	A L	L	A			L - bone marrow
	1	154							
	2	159							
VII	3	283							
To cast	4	283		L					
	5	291		L					
	6	291	L	L		L			
	7	325	A	A			A		